PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A01N 61/00, 37/42, 27/00, C12N 15/82, G01N 33/68

A2

(11) International Publication Number: **WO 98/00023**

(43) International Publication Date: 8 January 1998 (08.01.98)

(21) International Application Number: PCT/GB97/01672

(22) International Filing Date: 20 June 1997 (20.06.97)

(30) Priority Data:
9613753.4 1 July 1996 (01.07.96) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BROEKAERT, Willem, Frans [BE/BE]; Kluizenbosstraat 26, B-1700 Dilbeek (BE). THOMMA, Bart, Pierre, Helene, Joseph [NL/BE]; Raoul Claesstraat 1-17, B-3000 Leuven (BE). PENNINCKX, Iris, Anne, Marie, Armande [BE/BE]; Brugstraat 67, B-3001 Heverlee (BE). TERRAS, Franky, Raymond, Gerard [BE/BE]; Schaagstraat 96, B-8570 Anzegem (BE). MANNERS, John, Michael [AU/AU]; 28 Warmington Street, Paddington, QLD 4064 (AU). KAZAN, Kemal [TR/AU]; 1/24 Durham Street, St. Lucia, QLD 4072 (AU).

(74) Agents: KENT, Lindsey, Ruth et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: PLANT PROTECTION METHOD

(57) Abstract

A method of protecting a plant against a pathogen is described, the method comprising inducing expression of a plant defensin gene by stimulating the jasmonate and/or ethylene pathways. Also described is a method of inducing expression of a plant defensin gene, a composition which is capable of inducing expression of a plant defensin gene and a method for screening compounds giving resistance-inducing activity. Preferably, the pathogen is a necrotrophic pathogen.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

PLANT PROTECTION METHOD

The present invention relates to a method of protecting a plant against pathogens.

More particularly, the invention relates to a novel signal transduction pathway which leads to expression of proteins which are capable of protecting plants from attack by such pathogens.

In particular, the present invention relates to a method of protecting a plant against necrotrophic pathogens such as fungi and bacteria.

It is well known that plants attacked by microbial pathogens are able to induce the expression of a set of defence-related genes encoding pathogenesis-related proteins (PR proteins). These proteins can be detected not only at the site of pathogen attack but also in non-infected leaves and they give rise to systemic acquired resistance - a state in which the plant shows enhanced resistance to subsequent challenges by microbial pathogens. It is also known that salicylic acid (SA) plays an important role in the signal transduction pathway leading to systemic acquired resistance as infection of leaves by microbial pathogens causes an increase in the endogenous levels of salicylic acid followed by the induction of PR proteins.

The present invention relates to an alternative signal transduction pathway, the stimulation of which also appears to induce resistance to pathogens, especially microbial pathogens and which may be of use in addition to, or instead of, the salicylic acid pathway.

Previous work has demonstrated that exogenous application of jasmonate and methyl jasmonate on potato and tomato plants induces resistance against the late blight fungus *Phytophthora infestans* (Cohen *et al.* 1993). There is, however, no teaching of the protection of a plant against a pathogen by inducing expression of a plant defensin gene.

Treatment with methyl jasmonate did not confer resistance in either of two other plant/pathogen systems, namely barley/*Erysiphe graminis* (Kogel *et al.* 1995) or rice/*Magnaporthe grisea* (Schweizer *et al.* 1997).

It is well documented that compounds known to activate the salicylate-dependent pathogen-induced defence response, such as salicylic acid itself, acetylsalicylic acid, 2,6-dichloroisonicotinic acid (INA) and 1,2,3-benzothiadiazole-7-carbothioic acid S-methylester, can induce resistance to a range of microbial pathogens, including viruses, bacteria and fungi (White *et al.* 1979; Kessmann et al. 1994; Lawton *et al.* 1996; Friedrich *et al.*; Gorlach *et*

al.). Interestingly, 1,2,3-benzothiadiazole-7-carbothioic acid S-methylester failed to protect tobacco plants against infection by the necrotrophic pathogens *Botrytis cinerea* and *Alternaria alternata* (Lawton *et al.*).

Three *Arabidopsis* genes have previously been identified, namely *PR-1*, *PR-2* (β-1,3-glucanase) and *PR-5* (osmotin-like protein), that are coordinately and systemically induced upon pathogen infection (Uknes *et al.*, 1993; Dempsey *et al.*, 1993; Mauch-Mani and Slusarenko, 1994). These genes are all highly induced upon exogenous application of SA or INA, a synthetic compound that appears to mimic the action of SA (Uknes *et al.*, 1992; Cao *et al.*, 1994).

The present inventors have discovered that the induction of genes which encode protective proteins is not always dependent upon the salicylic acid pathway but may be related to a quite separate pathway

According to a first aspect of the present invention, there is provided a method of protecting a plant against a pathogen, the method comprising inducing expression of a plant defensin gene by stimulating the jasmonate and/or ethylene pathways.

According to a second aspect of the present invention, there is provided a method of inducing expression of a plant defensin gene by applying to the plant one or more of ethylene, jasmonate, an agent which mimics the action of ethylene or jasmonate and an agent which causes oxidative stress.

According to a third aspect of the present invention, there is provided a composition which is capable of inducing expression of a plant defensin gene comprising one or more of jasmonic acid, a jasmonate, ethylene, an agent which mimics the action of ethylene or jasmonate and an agent which is capable of causing oxidative stress.

According to a fourth aspect of the present invention, there is provided composition which is capable of inducing expression of a plant defensin gene comprising one or more of an ethylene-generating compound, a lipid derived signal molecule, salicylic acid, functional analogues of salicylic acid and reactive oxygen-generating compounds.

According to a fifth aspect of the present invention, there is provided a method for screening compounds for resistance inducing (defensin-inducing) activity, the method comprising applying to a plant or part of a plant a compound suspected of giving such

resistance and detecting the expression of a plant defensin gene or a co-ordinately expressed gene.

According to a sixth aspect of the present invention, there is provided a promoter which is capable of inducing the expression of a plant defensin gene comprising a region which is induced by jasmonic acid or an agent which mimics the action thereof and / or ethylene or an agent which mimics the action thereof.

According to a seventh aspect of the present invention, there is provided a promoter region comprised within the nucleic acid sequence shown in Figure 14, or a sequence that has substantial homology with that shown in Figure 14, or a variant thereof.

Preferably, the plant defensin gene is induced by stimulating the jasmonate and ethylene pathways.

Preferably, the pathogen is a necrotrophic pathogen.

Preferably, the pathogen is a microbial pathogen.

Preferably, the pathogen is a fungus.

Preferably, the jasmonate and/ or ethylene pathways are stimulated by the application of ethylene, jasmonic acid or a jasmonate. However, the application of an agent which mimics the action of ethylene or jasmonic acid would also be effective. Furthermore, it seems that expression of plant defensins can be induced by application of non-herbicidal amounts of agents which are capable of causing oxidative stress. Examples of such agents include diphenyl herbicides such as paraquat or diquat which result in the formation of superoxide anions or rose bengal which leads to the production of singlet oxygen species.

Preferably, the stimulation of the jasmonate and/ or ethylene pathways involves the signal transduction components EIN 2 and COI 1 from *Arabidopsis*. However, stimulation of these pathways may involve corresponding gene products in other plants which are substantially homologous to EIN 2 and COI 1.

Preferably, the ethylene-generating compound is selected from ethylene, ethephon and aminocyclopropanecarboxylic acid.

Preferably, the lipid-derived signal molecule is selected from arachidonic acid and derivatives thereof, linolenic acid and derivatives thereof and jasmonate and derivatives thereof.

Preferably, the reactive oxygen-generating compound is selected from paraquat, diquat, rose bengal and eosine.

Any plant species may be used but particularly suitable plants include radishes, tobacco or *Arabidopsis* species. When *Arabidopsis* species are used, the defensin may be the product of the plant defensin gene PDF 1.2 (Figure 14), a sequence that has substantial homology with the sequence of PDF 1.2, or a variant thereof.

As mentioned above, compounds which mimic the acitivity of jasmonate or ethylene will also induce the expression of plant defensin genes and this means that the pathway can be used to screen for compounds activating endogenous defence mechanisms.

Although whole plants may be used in the screen, it may be more convenient to use parts of plants, particularly tissues such as the leaves.

The detection may be carried out by any suitable means, for example by using antibodies against the gene products of PDF 1.1, or PDF 1.2, on a related plant defensin or by means of a reporter gene such as a GUS gene or a luciferase gene linked to the promoter region of PDF 1.2.

An advantage of the method of the present invention is that it does not involve the use of cytotoxic or potentially harmful chemicals that directly interfere with living microbial cells but makes use of chemicals that activate existing defence mechanisms in plants.

An advantage of the composition of the present invention is that it can be used to give a plant resistance against certain types of pathogens for instance against necrotrophic pathogens. Alternatively, the composition of the present invention may be used to give plants protection against a broad spectrum of pathogens by using compounds which induce the salicylic, jasmonate and/ or ethylene pathways.

A preferred embodiment of the present invention is a method of protecting a plant of the *Arabidopsis* species against a fungus, the method comprising inducing expression of a plant defensin gene by stimulating the jasmonate and/or ethylene pathways wherein expression of the defensin gene is induced by treatment of leaves with 0.5μM methyl jasmonate in an atmosphere containing 25 ppm ethylene.

An even more preferred embodiment of the present invention is a composition which is capable of inducing expression of a plant defensin gene in order to protect a plant against attack by a number of pathogens, wherein the composition comprises ethylene, methyl

jasmonate and salicylic acid. In this way, both the salicylate-dependent defence pathway and the jasmonate and/ or ethylene pathways may be activated.

The term "variant thereof" with reference to the present invention means any substitution of, variation of, modification of, replacement of, deletion of or the addition of one or more nucleotides from or to the gene sequence providing product of the resultant sequence is capable of anti-pathogenic activity. The term also includes sequences that can substantially hybridise to the gene sequence. It also includes DNA which hybridises to the DNA of the present invention and which codes for at least part of the gene sequence. Preferably, such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SSC at ambient temperature to about 65 °C, and high stringency conditions a 0.1 x SSC at about 65 °C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three times as concentrated as SSC and so on.

The term "substantial homology" covers homology with respect to at least the essential nucleotide/s of the gene sequence providing the homologous sequence acts as a defensin gene ie its product is capable of giving resistance against a pathogen of a plant. Typically, homology is shown when 60% or more of the nucleotides are common with the gene sequence of the present invention, more typically 65%, preferably 70%, more preferably 75%, even more preferably 80% or 855 and, especially preferred, are 90%, 95%, 98% or 99% or more homology.

The term "microbial" includes bacteria, fungi and viruses.

One of the genes that is activated in *Arabidopsis* upon microbial attack and whose expression is not dependent on salicylic acid but rather requires functional components of the ethylene and jasmonic acid response pathways is a plant defensin gene. Plant defensins are a family of cysteine-rich basic proteins of about 5 kDa in length which are structurally related to the antimicrobial insect defensins found in various insect species (Broekaert *et al* Plant, 1995). A number of these plant defensins were known to be potent inhibitors of fungal growth which suggested that they play a role in host defence. It has been shown that expression of a plant defensin gene from radish in transgenic tobacco plants confers resistance to the fungal pathogen *Alternania longipes* (Terras *et al.*, 1995). The pathogen induced plant defensin gene is most probably not the only gene activated via the jasmonate

and/or ethylene-dependent pathways ie a salicylic acid independent pathway, but it can be used as an indicator to monitor the activation of this pathway. However, any other gene operating in the same pathway could be used for the same purpose.

As described in greater detail in the examples below, two *Arabidopsis* genes encoding plant defensins were found to be expressed. These genes were found in a search of the Genbank database for sequences homologous to the nucleotide sequence for the cDNA of Rs-AFP1, a known radish plant defensin, and have the Genbank accesssion numbers Z27258 and T04323 and were designated *PDF1.1* and *PDF1.2* respectively. Their sequences are given in Figures 1A and 1B and as SEQ ID NOs1 and 2. These sequences were identified as expressed sequence tags in cDNA libraries prepared either from dry seed mRNA (*PDF1.1*) or seedling mRNA (*PDF1.2*). The genes encode preproteins with a putative peptide signal and a mature plant defensin domain which are 98% and 92% identical to the radish protein.

The expression patterns of *PDF1.1* and *PDF1.2* were analysed by reverse transcription polymerase chain reaction (RT-PCR) on DNAase-treated RNA isolated from different *Arabidopsis* organs (Figure 2). As a control for the RT-PCR reactions, a primer pair was designed for amplification of sequences corresponding to the region of the *Arabidopsis* ACTIN-1 gene (Nairn *et al,* Gene 1988) encompassing a 99 base pair intron. In this way, products derived from PCR amplification of genomic DNA can be discriminated by size from true RT-PCR products obtained from RNA. As can be seen in Figure 2, RNA samples from all analysed tissues, except dry seed, yielded *ACTIN-1* RT-PCR amplification products of the expected size, whereas genomic DNA yielded a PCR product which was about 100 bp longer. RT-PCR with a primer pair specific for *PDF1.1* showed amplification products with RNA from siliques and dry seed as templates. The primer pair specific for *PDF1.2* did not yield RT-PCR amplification products in any tisssue analysed from healthy plants. However, amplification products of the expected size were detected upon RT-PCR performed on RNA from leaves infected with *Alternaria brassicicola* strain MUCL 20297, a fungus causing brown necrotic lesions which do not spread over time. The PCR amplification product obtained with genomic DNA was about 100 bp longer than that obtained by RT-PCR on RNA from infected leaves, indicating that the *PDF1.2*-specific primers span an intron in the *PDF1.2* gene. This was not observed with *PDF1.1*-specific primers.

It was concluded that *Arabidopsis* contains two genes which encode highly homologous plant defensins but which have a totally different expression pattern. *PDF1.1* is expressed predominantly in seed and is considered to be the homologue of the radish Rs-AFP1 and RsAFP2 genes, whereas *PDF1.2* is expressed in leaves upon pathogen stress and can be considered as the homologue of the RsAFP3 and RsAFP4 genes from radish (Terras *et al.*,1995).

The *PDF1.1* and *PDF1.2* genes and their expression products were used in the further investigations which are described in more detail in the examples below.

Analysis of the expression of two plant defensin genes identified *via* their expressed sequence tag (EST) sequences revealed that they are differentially expressed and only one of these two genes, designated *PDF1.2*, appears to be pathogen-inducible. Although only two homologous plant defensin EST sequences have been identified so far, it is possible that other plant defensin genes may be present and possibly expressed in *Arabidopsis* because Southern blot analysis of *Arabidopsis* genomic DNA digested with four independent restriction enzymes and using a cDNA clone for *Rs-AFP2* as hybridisation probe revealed 3 to 5 bands depending on the enzyme used (data not shown). At present the expression pattern of other putative defensin genes during pathogenesis remains unknown and will require a careful examination of the gene family.

In the present work we have found that the expression of plant defensin genes, including at least *PDF1.2*, is also induced systemically upon pathogen infection but this induction follows a response pathway that is clearly different from that followed by PR-proteins. This finding is based on several lines of evidence.

First, *Arabidopsis* plant defensins were not induced upon external application of either SA or INA. In contrast, treatments with either methyl jasmonate, ethylene or the reactive oxygen species generating compounds paraquat and rose bengal caused accumulation of plant defensin transcripts. Second, the pathogen-induced systemic expression of plant defensin genes was not reduced in the *npr-1 Arabidopsis* mutant which is known to be blocked in the response pathway leading from SA to activation of PR-protein genes (Cao *et al.*, 1994). Third, plant defensin genes were not constitutively expressed in the *cpr1 Arabidopsis* mutant which displays constitutively elevated levels of endogenous SA as well as of *PR-1*, *PR-2* and *PR-5* transcripts (Bowling *et al.*, 1994). Fourth, analysis of the

etr1-3, *ein2* and *coi1* mutants of *Arabidopsis* demonstrated that the mechanism leading to induction of plant defensins in response to fungal challenge appears to involve components from or shared by the ethylene and jasmonate response pathways. These results therefore indicate that two separate classes of antifungal proteins (exemplified by PR-1 and plant defensin respectively) can be induced both locally and systemically by distinct signalling processes.

The *ein2 Arabidopsis* mutant, identified by a lack of morphological response when grown in the presence of ethylene (Guzman and Ecker, 1990) is virtually blocked in its pathogen-induced expression of plant defensin genes both in pathogen-treated leaves and in non-treated, systemic leaves. In contrast, the *etr1-3* mutant, which is a partially ethylene-insensitive mutant (Chang *et al.*, 1993), had a normal plant defensin response in infected leaves but exhibits reduced but not abolished plant defensin gene expression in systemic leaves of infected plants. The incomplete suppression of plant defensin gene expression is pathogen-challenged *etr 1*-3 plants is most probably due to the leakiness of this particular mutant allele. The *coi1* mutant is known to be less sensitive than wild-type plants to inhibition of root growth upon treatment with methyl jasmonate or coronatine, a bacterial phytotoxin acting as a jasmonate analog (Feys *et al.*, 1994). This mutant showed an almost completely blocked pathogen-induced plant defensin response both in the pathogen-treated and non-treated, systemic leaves.

From these analyses it thus appears that EIN2 and COI1 are required for local as well as systemic plant defensin induction, whereas *ETR 1* appears to be involved only in the systemic response. Of these three genes, only *ETR 1* has been identified. *ETR 1* encodes a protein resembling bacterial two-component histidine kinase sensors, and genetic and biochemical evidence indicates that ETR 1 is an ethylene receptor (Schaller and Bleecker, 1995). The gene product EIN2 acts downstream of ETR 1 in the ethylene response pathway (Ecker, 1995). COI1 has not been characterized to date but it is believed to be involved in signal transduction initiated by jasmonates (Feys *et al.*, 1994).

Interestingly, *ein2* plants have previously been found to display decreased chlorotic lesion formation relative to wild-type plants when infiltrated with virulent strains of the bacterium *Pseudomonas syringae* pv. *tomato* (Bent *et al.*, 1992). Mutants carrying the *etr1-3* mutation (previously called *ein1-1*) responded like wild-type plants. Although *ein2* plants

showed decreased disease symptoms relative to wild-type and *etr1-3* plants, the *P. syringae* pv. *tomato* bacteria multiplied equally well in these three genotypes.

A model for two separate pathways leading to induction of defence-related genes upon pathogen stress is presented in Figure 9. In this model, the hypersensitive response is positioned above the bifurcation point, because *acd2 Arabidopsis* mutants developing spontaneous hypersensitive response-like lesions (Greenberg *et al.*, 1994) were found to contain enhanced transcript levels of both plant defensins and PR-protein (Greenberg *et al.*, 1994). The pathway leading to PR-protein gene expression *via* salicylic acid involves the signal transduction components NPR1 and CPR1, whereas the pathway leading to plant defensin gene expression would require EIN2 and COI1. There may also be some degree of "cross-talk" between both defence response pathways and it appears that inhibition of SA accumulation results in enhanced activation of the jasmonate and/ or ethylene pathways ie SA-independent pathway. In this regard, acetylsalicylic acid was shown to inhibit jasmonate biosynthesis (Peña-Cortes *et al.*, 1993), whereas SA was found to inhibit the induction of proteinase inhibitors by jasmonic acid (Doares *et al.*, 1995).

Other defence-related genes may be activated coordinately with plant defensins *via* the SA-independent pathway depicted above. A first likely candidate is *Hel*, a hevein-like (*PR*-4-like) gene that is induced both locally and systemically upon viral infection (Potter *et al.*, 1993). *Hel* is strongly induced by ethylene but only weakly by SA, whereas *PR-1*, *PR-2* and *PR-5* are not ethylene-inducible but strongly SA-inducible (Potter *et al.*, 1993). A second candidate is the thionin gene *Thi2.1* which is induced upon fungal infection and methyl jasmonate treatment, but not upon SA treatment (Epple *et al.*, 1995). A third possible candidate is the basic chitinase gene *CHIT-B* which is induced upon ethylene treatment in wild-type plants but not in *ein2* or *etr1-3* mutants (Chen and Bleecker, 1995). It was observed that the induction of *CHIT-B* in virus-infected leaves of *Arabidopsis* followed different kinetics relative to the induction of *PR-1*, *PR-2* and *PR-5* (Dempsey *et al.*, 1993).

There is also evidence for the existence of two separate pathways for pathogen-inducible responses in tobacco. The first pathway leads to induction of acidic PR-protein genes such as *PR-1*, *PR-2*, *PR-3* (acidic chitinase), *PR-4* and *PR-5*, whereas the second pathway results in induced expression of the basic β-1,3-glucanase and basic chitinase genes. The first group of genes is strongly activated by SA, while the second group responds to

ethylene (Meins *et al.*, 1991). Interestingly, transgenic tobacco plants expressing a gene encoding the A1 subunit of cholera toxin, a G-protein inhibitor, showed constitutive expression of the acidic PR-protein genes but not of the basic β-1,3-glucanase and basic chitinase genes (Beffa *et al.*, 1995). The acidic PR-protein genes are induced both locally and systemically upon challenge with tobacco mosaic virus (TMV) (Ward *et al.*, 1991; Brederode *et al.*, 1991). The basic β-1,3-glucanase and basic chitinase genes, on the other hand, are also strongly induced in the inoculated leaves but there is contradictory evidence as for their systemic inducibility. Based on RNA blot analysis, no significantly enhanced transcript levels of these genes could be detected in uninoculated leaves of TMV-infected plants (Ward *et al.*, 1991; Brederode *et al.*, 1991), whereas the corresponding proteins were found to accumulate in such leaves based on Western blot analyses (Heitz *et al.*, 1994).

It may be speculated that pathogen-induced expression of the basic β-1,3-glucanase and basic chitinase genes in tobacco follows a pathway equivalent to that leading to induced plant defensin gene expression in *Arabidopsis*. One apparent difference is that induced expression of plant defensins occurs in systemic leaves both at the transcript level and at the protein level. It should be taken into account that *Arabidopsis* is much smaller than tobacco and hence that systemic responses in the former are measured over much shorter distances. Another obvious difference between basic β-1,3-glucanase and basic chitinase expression in tobacco on the one hand, and plant defensin gene expression in *Arabidopsis* on the other hand, is that the tobacco genes are wound-inducible (Brederode *et al.*, 1991), whereas plant defensin genes in *Arabidopsis* are not.

We have demonstrated that a pathogen-induced protein cross-reacting with antibodies raised against a PDF1.2 analog from radish (Rs-AFP1) possesses strong antifungal activity *in vitro*. This protein accumulates to rather high levels upon fungal infection, namely up to 2% and 1% of total proteins in inoculated and untreated, systemic leaves, respectively. We have previously shown that transgenic tobacco plants producing a PDF1.2 analog from radish (Rs-AFP2) at about 0.25% of total soluble proteins are more resistant to *A. longipes* than untransformed plants (Terras *et al.*, 1995). Based on these observations it is likely that plant defensins have a role to play in host defence. There is now compelling evidence that a SA-dependent pathway is important for mounting resistance to some pathogens, including *P. syringae* pv. *tomato* and *Peronospora parasitica*. Indeed, *nahG*-expressing plants were

found to be considerably more sensitive to these pathogens relative to wild-type plants (Delaney *et al.*, 1994) and, unlike wild-type plants, were unable to mount systemic acquired resistance (Lawton *et al.*, 1995). Evidence is presented here to show that the SA-independent pathway (leading to activation of plant defensin genes, and possibly other defence-related genes as well) also contribute to resistance, albeit to different types of pathogens. Indeed, we have shown that the *ein* 2 and *coi* 1 mutants, which are apparently blocked in the SA-independent pathway, are more susceptible to infection by *Botrytis cinerea* than wild type plants.

The invention will now be further described only by way of example in the following examples and in the Figures in which:-

Figure 1 shows nucleotide sequences and deduced amino acid sequences of expressed sequence tags Z27258 and T04323 corresponding to *PDF1.2* and *PDF1.2*, respectively.

(A) Nucleotide sequence and deduced amino acid sequence of Z27258 corresponding to *PDF1.1*. The experimentally determined N-terminal region of PDF1.1 previously called At-AFP1 (Terras *et al.*, 1993) is underlined. The nucleotide at position 123 (T in Z27258) has been replaced by a G according to additional sequence data.

(B) Nucleotide sequence and deduced amino acid sequence of T04323 corresponding to *PDF1.2*.

(C) Alignment of the amino acid sequences of mature Rs-AFP1 and Rs-AFP3 from radish seed and leaves, respectively (Terras *et al.*, 1995; F.R.G. Terras, unpublished results) with the mature domain of PDF1.1 and the putative mature domain of PDF1.2.

Underlined nucleotide sequences in (A) and (B) correspond to the positions of primers used for RT-PCR. Stop codons in (A) and (B) are double underlined.

Figure 2 shows the expression pattern of *PDF1.1* and *PDF1.2* in *Arabidopsis*;

(A) RT-PCR analysis using a primer pair specific for *PDF 1.1*.

(B) RT-PCR analysis using a primer pair specific for *PDF 1.2*.

(C) RT-PCR analysis using a primer pair specific for *ACTIN-1*.

RT-PCR reactions were performed on DNase-free total RNA isolated from different *Arabidopsis* organs. Roots, stems, flower buds, open flowers and siliques were collected from 7-week-old flowering plants. Leaves and infected leaves were collected from 4-week-

old plants. Infected leaves were inoculated with *A. brassicicola* and collected after 3 days of incubation.

Figure 3 shows purification and characterisation of a plant defensin from infected *Arabidopsis* leaves;

(A) Separation of the basic protein fraction of healthy *Arabidopsis* leaves (upper part) and *A. brassicicola*-infected *Arabidopsis* leaves (lower part) on a C2/C18 silica reversed-phase chromatography column. The column was eluted with a linear gradient from 0% to 50% (v/v) of acetonitrile in 0.1% (v/v) trifluoroacetic acid.

(B) Electrophoretic analysis of proteins contained in peak 1. Two hundred ng of protein contained in peak 1 and 200 ng of purified Rs-AFP2 were electrophoresed on an SDS-PAGE gel (Phastgel High Density, Pharmacia) and silver stained. Sizes of the molecular mass markers are indicated at left in kilodaltons.

(C) Immunoblot of proteins contained in peak 1 after SDS-PAGE using anti-Rs-AFP1 antibodies. Lanes were loaded with either 200 ng of purified Rs-AFP2 or 200 ng of purified protein contained in peak 1.

(D) In vitro antifungal activity against *A. brassicicola* of water (negative control, left panel), purified Rs-AFP2 at 5 µg/mL (middle panel) or 5 µg/mL purified protein contained in peak 1 (right panel). Micrographs were taken after 24h of incubation at 22°C.

Figure 4 shows expression of plant defensins in *Arabidopsis* upon fungal infection;

(A) RNA gel blot analysis of *PDF1.2* and β-*Tubulin* (β-*TUB*) expression in pathogen-treated leaves (1°) and non-treated, systemic leaves (2°) of infected plants. All analyzed samples represent 4 µg of total RNA.

(B) Plant defensin (PDF) content in pathogen-treated leaves (circles) and non-treated, systemic leaves (squares) of infected plants as determined by ELISA using antigen affinity-purified anti-Rs-AFP1 antiserum. Values are means (± standard error) from three independent determinations.

Total RNA and proteins were isolated from pathogen-treated and non-treated, systemic *Arabidopsis* leaves collected 0h, 3h, 6h, 12h, 24h, 48h, 72h and 96h after inoculation with 5 µL drops (5 drops per leaf) of *A. brassicicola* spores at $5x10^5$ spores/mL.

Figure 5 shows induction of plant defensins in *Arabidopsis* leaves upon chemical treatments and wounding;

(A) RNA gel blot analysis of *PDF1.2* and β-*Tubulin* (β-*TUB*) expression in leaves wounded or treated with the indicated chemicals. All analyzed samples represent 4 µg of total RNA.

(B) Plant Defensin (*PDF*) content as determined by ELISA using antigen affinity-purified anti-Rs-AFP1 antiserum. Values are means (± standard error) of three independent determinations.

Arabidopsis leaves were inoculated with 5 µL drops (5 drops per leaf) of water, SA (5 mM), INA (1 mg/mL), paraquat (25 µM), rose bengal (20 mM), methyl jasmonate (45 µM in 0.1% (v/v) ethanol) or 0.1% (v/v) ethanol (0.1% EtOH). Ethylene treatment was performed by placing plants in an air-tight chamber with an ethylene concentration of 20 ppm. Control plants (air) were incubated in an identical chamber without ethylene. Wounding was applied by making incisions in the leaf with a scalpel. All leaf samples were collected 48h after initiations of the treatments. The experiment was repeated once with similar results.

Figure 6 shows induction of plant defensins in *Arabidopsis* wild-type (Col-0), and in *Arabidopsis* mutants (*npr1* and *cpr1*) affected in the SA-signalling pathway;

The left part of the figure shows RNA gel blot analyses of *PDF1.2* expression. The samples represent 4 µg of total RNA.

The right part shows plant defensin (PDF) contents as determined by ELISA using antigen affinity-purified anti-Rs-AFP1 antiserum. Values are means (± standard error) of three independent determinations.

Arabidopsis plants were inoculated with *A. brassicicola* (A. bras.) by applying 5 µL drops of a spore suspension ($5x10^5$ spores/mL) on four lower rosette leaves (5 drops per leaf). Control plants were treated identically with 5 µL drops of water (H_2O). Pathogen-treated leaves (1°) and non-treated leaves of the same plants (2°) were collected 3 days after inoculation. Total RNA and proteins were extracted as described (see methods). The experiment was repeated twice with similar results.

Figure 7 shows induction of plant defensins in *Arabidopsis* wild-type (col-0), in *Arabidopsis* mutants (*ein2* and *etr1-3*) affected in the ethylene response pathway and in a mutant (*coi1*) affected in the jasmonate response pathway.

The left part of the figure shows RNA gel blot analyses of *PDF1.2* expression. The samples represent 4 µg of total RNA.

The right part shows plant defensin (PDF) contents as determined by ELISA using antigen affinity-purified anti-Rs-AFP1 antiserum. Values are means (± standard error) of three independent determinations.

Arabidopsis plants were inoculated with *A. brassicicola* (A. bras.) by applying 5 µL drops of a spore suspension ($5x10^5$ spores/mL) on four lower rosette leaves (5 drops per leaf). Control plants were treated identically with 5 µL drops of water (H_2O). Pathogen-treated leaves (1°) and non-treated leaves of the same plants (2°) were collected 3 days after inoculation. Total RNA and proteins were extracted as described (see methods). The experiment was repeated twice with similar results.

Figure 8 shows induction of plant defensins in *Arabidopsis* wild-type (Col-0) and in an *Arabidopsis* lesion mimic mutant (*acd2*).

(A) RNA gel blot analysis of *PDF1.2* expression. All analyzed samples represent 4 µg of total RNA.

(B) Plant Defensin (PDF) content as determined by ELISA using antigen affinity-purified anti-Rs-AFP1 antiserum. Values are means (± standard error) of three independent determinations.

Total RNA and proteins were isolated from healthy asymptomatic upper rosette leaves (UH) and lower rosette leaves displaying necrotic lesions (LN) collected from 5-week-old *acd2*-plants, as well as from healthy upper rosette (UH) and lower rosette leaves (LH) from control plants (Col-O) grown under identical conditions. The experiment was repeated twice with similar results.

Figure 9 is a proposed model for the induction of defence-related genes via two separate pathways, namely a salicylate-dependent pathway and a jasmonate and/or ethylene-dependent pathways.

Figure 10 shows three alternative models for the interaction between ethylene and jasmonate signals during activation of the *PDF1.2* gene in pathogen-stressed *Arabidopsis* plants.

Figure 11 shows a time course of jasmonic acid levels in *Arabidopsis* wild-type plants (Col-0, upper panel) and ethylene-insensitive mutants (*ein2*, lower panel) upon inoculation with *Alternaria brassicicola* (closed symbols) or mock inoculation with water

(open symbols). Each datapoint is the average of two separate measurements on two sets of three plants each.

Figure 12 shows a time course of ethylene production levels in *Arabidopsis* wild-type plants (Col-0, upper panel) and jasmonate-insensitive mutants (*coi1*, lower panel) upon inoculation with *Alternaria brassicicola* (closed symbols) or mock inoculation with water (open symbols). Data for Col-0 are averages of three independent experiments with two plants for each time point. Data for *coi1* are from a single experiment with two plants for each time point.

Figure 13 shows the induction of plant defensins (PDF) in *Arabidopsis* wild type plants (Col-0) and ethylene-insensitive mutants (*ein2*) upon treatment with 0.1% ethanol (con) or 50 μM methyl jasmonate in 0.1 % ethanol (MeJA).

Figure 14 shows the nucleotide sequence of the *Arabidopsis PDF1.2* gene. The boxed nucleotide represents the first nucleotide of expressed sequence tag T04323. The amino acids of the gene product are shown below the corresponding codons of the coding region. The intron is shown in lower case letters.

Figure 15 shows β-glucuronidase activity in transgenic pPDF1.2-GUS-tNOS *Arabidopsis* plants upon inoculation with *A. brassicicola* (mock or spore-inoculated) or *B. cinerea* (mock or spore-inoculated). Treated leaves (1°) and non-treated leaves (2°) of the same plant were collected 3 days after treatment. Results are expressed as averages ± standard errors of four sets of two plants.

Figure 16 shows β-glucuronidase activity in transgenic pPDF1.2-GUS-tNOS *Arabidopsis* plants (panel A) and transgenic pBgl2-GUS-tNOS *Arabidopsis* plants (panel B) upon treatment with various chemicals. Samples of treated leaves were collected 48 h after treatment. Results are expressed as averages ± standard errors of four individually harvested plants.

Figure 17 shows β-glucuronidase activity in transgenic pPDF1.2-GUS-tNOS tobacco (cv. Xanthi-nc) plants with tobacco mosaic virus or mock-inoculated. The leaves just below the youngest fully expanded leaves were either virus- or mock-inoculated. Those leaves (1°), the youngest fully expanded leaves (2°) and the leaves just above the youngest fully expanded leaves (3°) were harvested separately at two days (black bars), 4 days (light grey bars) or 6 days (dark grey bars) following treatment.

Figure 18 shows β-glucuronidase activity in transgenic pPDF1.2-GUS-tNOS tobacco (cv. Xanthi-nc) plants upon wounding or treatment with various chemicals. Samples of treated leaves were harvested 48 h after treatment.

Figure 19 shows β-glucuronidase activity in transgenic pPDF1.2-GUS-tNOS *Arabidopsis* plants upon exposure to 25 ppm ethylene, treatment with 0.5 μM methyl jasmonate (MeJA, in 0.1% ethanol), 333 μM ethephon, 25 ppm ethylene plus 0.5 μM methyl jasmonate, 333 μM ethephon plus 0.5 μM methyl jasmonate, and the appropriate control treatments: air exposure and treatment with water and 0.1% ethanol.

Figure 20 shows the decay of *Arabidopsis* wild type plants (Col-0, circles), ethylene insensitive mutants (*ein2*, triangles) and jasmonate insensitive mutants (*coi1*, squares) after inoculation with the fungal pathogen *Botrytis cinerea*. Data represent averages with standard deviations of four independent experiments (Col-0 and *ein2*) and three independent experiments (*coi1*) performed with series of 20 plants for each plant line.

Figure 21 shows lactophenol/trypan blue staining of hyphal structures of the fungus *Peronospora parasitica* pathovar Wela in leaves of inoculated *Arabidopsis* wild type plants (Col-0) and *ein2*, *coi1* and *npr1* mutants.

METHODS

Biological Material

The mutants *npr 1* (Cao *et al.*, 1994) and *cpr 1* (Bowling *et al.*, 1994) were provided by Dr. X. Dong (Duke University, Durham, NC, USA). The ethylene response mutants *ein2* (Guzman and Ecker, 1990) and *etr1-3* (Bleecker *et al.*,1988; Chang *et al.*, 1993) were obtained from the *Arabidopsis* Biological Resource Centre, OH, USA (accession number CS3071 and CS3070, respectively) and the lesion mimic mutant *acd2* (Greenberg *et al.*, 1994) was provided by Dr. F. Ausubel (Massachusetts General Hospital, Boston, USA). The jasmonate response mutant *coi1* (Feys *et al.*, 1994) was obtained from Dr. J. Turner (University of East Anglia, Norwich, UK). Since this mutation is recessive and causes male sterility, *coi1* mutants were identified *a posteriori* in F2 plants grown from seed from selfed *COI1/coi1* hemizygous plants. Therefore, the F2 population was subjected to different treatments as indicated below, leaves from each individual collected separately and the plants further grown untill seed set. Individuals that did not form siliques were identified as having the *coi1/coi1* genotype. The *coi* 1 mutants used for the disease assays had been preselected based on root length of young seedlings germinated *in vitro* in the presence of 50 μM methyl jasmonate. All mutant lines listed above are derived from the Columbia (Col-O) ecotype. Growth and spore harvesting of the fungus *A. brassicicola* (MUCL 20297; Mycothèque Université Catholique de Louvain, Louvain-la-Neuve, Belgium) was done as described previously (Broekaert *et al.*, 1990).

Plant Growth Conditions, Chemical Application and Inoculation

Arabidopsis seed were sown on flower potting compost containing a macro-nutrient supplement (Asef. Didam, The Netherlands) in petri-dishes. The seed were vernalized for 2 days at 4°C following sowing. After 5 days of incubation in a growth chamber (20°C day temperature, 18°C night temperature, 12-hr photoperiod at a photon flux density of 100 μE $m^{-2}s^{-1}$), seedlings were transferred to pots (5x4x4 cm) containing potting compost supplemented with macro-nutrients and grown under the same conditions as above. Irrigation was done with tap water *via* the trays carrying the pots. Leaves were wounded by crushing with forceps with ribbed tips or by making incisions in the limb with a scalpel taking care to leave the midvein intact. Paraquat (25 μM), rose bengal (20 mM), methyl jasmonate (45 μM

in 0.1% (v/v) ethanol), 0.1% (v/v) ethanol, sodium salicylate (5 mM) and INA (1 mg/mL of a 25% active ingredient in a wettable powder, provided by Dr. H. Kessmann, Novartis, Basel, Switzerland) were applied at the concentrations indicated between brackets as 5 µL droplets on leaves (5 drops per leaf). The stock solution of methyl jasmonate was 45 mM in ethanol. Ethylene treatment was performed by placing pots in an air-tight translucent chamber in which gaseous ethylene was injected *via* a silicon rubber septum. The ethylene concentration in the chamber was verified by gas chromatography. Control plants for the ethylene experiment were placed in an identical chamber without ethylene. Inoculation with *A. brassicicola* was done by applying 5 µL drops of a spore supension (density of $5x10^5$ spores/mL in distilled water) on four lower rosette leaves (5 drops per leaf). Control plants were treated identically with water droplets. The plants with drops of spore suspension or water were placed randomly (if different genotypes were treated simultaneously) in a propagator flat with a clear polystyrene lid and kept at high humidity for 2 days to stimulate infection by hyphal germlings. Thereafter, lids were taken off and the plants incubated further till harvesting of leaf material. The isolate of *A. brassicicola* and inoculation conditions used here caused limited brown necrotic lesions under the drops of spore suspension within 48h of inoculation and these lesions failed to spread further.

RNA Blot Analysis

RNA was extracted by the phenol/LiCl method according to Eggermont *et al.* (1996) from tissues frozen in liquid nitrogen and stored at -80°C. RNA samples were loaded at 4 µg per lane on a formaldehyde-agarose gel and blotted onto a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany) *via* capillary transfer with 20x SSC (Sambrook *et al.*, 1989). To verify equal and transfer of RNA, the loading buffer was supplemented with 50 µg/mL ethidium bromide, allowing visualization of RNA in the gels and on the blots upon UV illumination. RNA was cross-linked on the blots by UV illumination of both sides for 5 minutes each. Blots were prehybridized, hybridized with digoxigenin-labeled antisense RNA probes and developed by immunochemiluminescence as described before (Terras *et al.*, 1995). Digoxigenin-labeled probes were made by run-off transcription using the Dig RNA labeling kit of Boehringer Mannheim. The *PDF1.2* probe was synthesized using SP6 RNA polymerase and the *Eco*RI-linearized plasmid pZL1 (BRL

life Technologies, Gaithersburg, MD, USA) containing the expressed sequence tag with Genbank accession number T04323. The probe for the tubulin β-1 chain gene was synthesized using T7 RNA polymerase and the *Eco*RI-linearized plasmid pBluescript II SK (Stratagene, La Jolla, CA, USA) containing the expressed sequence tag with Genbank accession number Z26191. Both plasmids were obtained from the *Arabidopsis* Biological Resource Centre (Columbus, OH, USA). Samples analyzed with different probes were run on replicate gels which were developed separately.

Reverse Transcription PCR

Total RNA (1 μg in 100 mM sodium acetate/5 mM $MgSO_4$, pH 5.0) was treated with 6 units of RNase-free DNaseI (Boehringer Mannheim) at 37°C for 5 minutes, whereafter the DNase was inactivated by heating at 95°C for 5 minutes. The RNA was precipitated overnight in the presence of 0.2 M sodium acetate and 66% (v/v) ethanol, collected by centrifugation at 10000 x*g* for 10 minutes, washed twice with 70% (v/v) ethanol and finally dissolved in RNase-free water. The reverse transcription reactions were performed on 1 μg of DNase-treated total RNA with 10 units of avian myeloblastosis virus reverse transcriptase (Pharmacia, Uppsala, Sweden) for 60 minutes at 52°C. The reverse transcription reactions were performed with a homopolymeric deoxythymidine oligonucleotide (20-mer) and terminated by addition of Na-EDTA to a final concentration of 15 mM. A fraction (one thirtieth) of the reverse transcription reaction solution was used as a template in a 50 μL PCR reaction performed with 2.5 units of Taq polymerase (Appligene, Pleasanton, CA, USA) according to Sambrook *et al.* (1989). The PCR was run for 30 cycles with an annealing temperature of 55°C, 65°C and 65°C for amplification with primer pairs specific for *PDF1.1*, *PDF1.2* and *ACTIN-1*, respectively. Primers used for amplification of *PDF1.1* were:
OWB260 (sense 5'-GAGAGAAAGCTTGTTGTGCGAGAGGCCAAGTGGG-3'); and
OWB259(antisense 5'-GAGAGAGGATCCTGCAAGATCCATGTCGTGCTTTC-3'),
those for amplification of *PDF1.2* were:
OWB240 (sense, 5'-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3'); and
OWB241 (antisense, 5'-AATCCATGGAATACACACGATTTAGCACC-3');
and those for amplification of *ACTIN-1* were:
OWB270 (sense, 5'-GGCGATGAAGCTCAATCCAAACG-3'); and

OWB271 (antisense, 5'-GGTCACGACCAGCAAGATCAAGACG-3').

Purification of Plant Defensins from *A. brassicicola*-Infected *Arabidopsis* Leaves

Leaves of 5-week-old *Arabidopsis* plants were inoculated with 5 µL drops of distilled H_2O (control) or a *A. brassicicola* spore suspension ($5x10^5$ spores/mL in H_2O) and collected after 3 days of incubation in a moist propagator flat with a clear polysterene lid. Extracts were prepared from 20 g of either H_2O-treated or inoculated leaves and subjected to the purification procedure exactly as previously described in Terras *et al.* (1995). Protein determination, in vitro antifungal activity determination and SDS-PAGE analysis on precast PhastGel High Density gels (Pharmacia) were performed as previously described (Terras *et al.*, 1995). Prior to SDS-PAGE analysis, protein samples were reduced with dithioerythritol and S-pyridylethylated as in Terras *et al.* (1992). Immunoblotting of proteins, separated on a 15% acrylamide SDS-PAGE gel was done as described in Terras *et al.* (1995).

ELISA Analysis

Proteins were isolated from frozen leaf material in extraction buffer (10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 1.5% (w/v) polyvinylpolypyrrolidone, pH 7). Protein concentrations were determined in the crude extracts according to Bradford (1976) using bovine serum albumin as a standard. After heat treatment (10 min, 80°C) of the extract the heat-stable soluble protein fraction was analyzed in a competition ELISA.

ELISA Microtiterplates (Greiner Labortechnik) were coated with 100 ng/mL Rs-AFP2 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) for 2h at 37°C. The uncoated sites were blocked with 3% (w/v) cold fish skin gelatine (Sigma) in phosphate buffered saline (PBS) (2h, 37°C). Affinity-purified primary antibodies were diluted 50-fold in 0.3% (w/v) gelatine in PBS, containing 0.05% (v/v) Tween20 and applied to the wells simultaneously with equal volumes (50 µL) of the samples diluted in the sample bufer. The plates were incubated for 1h at 37°C. After several washes with PBS containing 0.1% (v/v) Tween20, the plate wells were filled (100 µL per well) with secundary antibodies (goat anti-rabbit antibodies coupled to alkaline phosphatase, Sigma Immuno Chemicals) diluted 1000 fold in 0.3% (w/v) gelatine in PBS, containing 0.05% (v/v) Tween20. The plates were incubated for 1h at 37°C. Alkaline phosphatase activity was measured after 30 to 60 min of

incubation at 37°C using the substrate 4-nitrophenyl phosphate (Janssen Chimica, Beerse, Belgium) at a concentration of 1 mg/mL in substrate buffer (20 mM Na_2CO_3, 35 mM $NaHCO_3$, 5 mM $MgCl_2$, pH 9.6) by absorbance measurement at 405 nm. Protein samples were applied in 4-fold dilution series prepared in triplicate and the plant defensin concentration was measured relative to a two-fold dilution series of purified Rs-AFP1 (Terras *et al.*, 1992) as a standard.

Affinity purification of anti-Rs-AFP1 antiserum was done as follows. An antigen affinity column was prepared by mixing equal volumes of 20 mg/mL purified Rs-AFP1 in 100 mM 3-N-morpholinopropanesulfonic acid buffer (pH 7) with Affi-Gel 10 matrix (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated in water. The slurry was incubated overnight at 4°C with continuous gentle agitation. After blocking the unreacted sites of the matrix by addition of 0.025 volumes of 1 M ethanolamine (pH 8), the column was subsequently washed with 10 mM Tris (pH 7.5), 100 mM glycine (pH 2.5), 10 mM Tris (pH 8.8) and 100 mM triethylamine (pH 11.5), and equilibrated with 10 mM Tris (pH 7.5). The rabbit anti-Rs-AFP1 antiserum (Terras *et al.*, 1995) was diluted two-fold in ImmunoPure Gentle Ag/Ab binding buffer (Pierce Chemical Company, Rockford, IL, USA) and passed several times over the affinity column. After washing the column with 15 volumes of ImmunoPure Gentle Ag/Ab binding buffer, purified anti-Rs-AFP1 antibodies were eluted with ImmunoPure Gentle Ag/Ab elution buffer (Pierce Chemical Company). The elution of bound antibodies was monitored by measuring the absorbance at 280 nm. The eluted fraction was dialyzed overnight against PBS.

Amplification, Cloning and DNA Sequence Analysis of the Promoter Region of the *Arabidopsis PDF1.2* Gene.

A strategy based on the inverse polymerase chain reaction (for details of this method see Gasch *et al.* 1992) was used to amplify 5' regulatory sequences of the *PDF1.2* gene. The DNA sequence of the expressed sequence tag (EST) with Genbank accession number T04323 that corresponds to the *PDF1.2* cDNA was used to design primers for the amplification of 5' genomic flanking sequences from the *Arabidopsis* genome. Total genomic DNA was isolated from 10 g fresh weight of leaves from approximately 8 week old plants of *Arabidopsis* ecotype Col-0 using the method of Dellaporta et al. (Dellaporta *et al.*). The

DNA was further purified by resuspending in a CsCl solution at a final density of 1.55 g/ml and containing 0.75 mg/ml of ethidium bromide and centrifuging at 45.000 rpm. The banded DNA was then removed from the centrifuge tube by a syringe, the ethidium bromide removed by partitioning against isoamyl alcohol and the DNA then precipitated by ethanol. The precipitated DNA was dissolved in water and 120 ng digested for 1 h at 37 °C with 10 units of the restriction enzyme Sph1 in a 40 µl reaction. The EST T04323 was known to have an internal Sph1 site (at bases 174 to 179) and therefore this enzyme was used to excise genomic fragments that would contain the first 178 bases of the cDNA, any intervening sequences and any 5' sequence upstream of the EST sequence to the first Sph1 site in the genomic DNA. The reaction was heat inactivated at 65 °C for 10 min, centrifuged briefly and the DNA precipitated from the supernatant using ethanol. Approximately 30 ng of the digested DNA was then self-ligated overnight at 14 °C in a standard 50 µl reaction using 1 unit of T4 DNA ligase and a buffer provided by the enzyme supplier (Boehringer Mannheim). The ligation reaction was stopped by heating at 65 °C for 10 min, briefly centrifuged and the DNA precipitated from the supernatant using ethanol. A sample of 10 ng of the ligated DNA was then added as template to a 50 µl Polymerase Chain Reaction (PCR) containing 200 µM dNTPs and 1 µM of each of the primers OWB257 [5' - GAGAGAGGATCCAACTTCTGTGCTTCCACCATTGC - 3', BamHI site underlined] and OWB256 [5' - GAGAGAAAGCTTGAAGCCAAGTGGGACATGGTCAGG - 3', HindIII site underlined]. These primers correspond to sequences at positions 104 - 127 and 133 - 156 in the EST T04323 sequence, respectively, but are inverted and would only amplify if flanking sequences were aligned by self-ligation of the genomic fragment at the terminal Sph1 sites. The PCR reaction contained 1 unit of Taq DNA polymerase (added when the reaction reached 95 °C in the first thermal cycle) and the reaction buffer recommended by the supplier (Appligene Inc.). The PCR reaction was subjected to the following thermal cycle regime. First, heating at 95 °C for 4 min during which the Taq polymerase enzyme was added, this followed by 30 cycles of 30 sec at 95 °C, 2 min at 56 °C (annealing temperature) and 1 min at 72 °C, and a final cycle of 30 sec at 95 °C, 2 min at 56 °C and 10 min at 72 °C. A sample equivalent to 0.05 µl of this reaction was added as template to a second PCR reaction that was identical except that no genomic DNA was added and that the nested primer OWB255 [5'-GAGAGAGGATCCAGCAGCAAAGAGAACAAGAGCAGCG - 3',

BamHI site underlined] was added instead of primer OWB257. The primer OWB255 corresponds to positions 67 - 91 in the T04323 sequence. This reaction was subjected to the same thermal cycling regime except that the 56 °C step was increased to 58 °C. Two DNA fragments approximately 1.3 kb and 0.8 kb in size were obtained. The larger band was isolated from the agarose gel, digested with HindIII and BamHI and ligated into pEMBL18+ predigested with HindIII and BamHI. This clone was termed pJMiPCR-1t. The insert was partially sequenced using dideoxynucleotide teminators and the M13 forward and M13 reverse primers. These partial sequences revealed that the insert contained sequences identical to those that would be predicted to be present from the EST T04323 sequence and partial sequences that flanked the Sph1 restriction site used for the self-ligation were identified. An oligonucleotide primer was then designed to the sequence adjacent to the Sph1 site in the pJMiPCR-1t insert that was predicted to contain sequences located in the 5' promoter region of the *PDF1.2* gene. This primer was termed OWB273 [5' - AAGAAAGCTTATGCATGCATCGCCGCATCGATATCCC - 3', HindIII site underlined]. This primer was used in a PCR reaction in combination with primer OWB276 [5' - GAGAGAAGCTTATTTTTATGTAAAATACACACGATTTAGCACC - 3', HindIII site underlined] which contains sequence corresponding to positions 292 - 326 in the EST T04323 sequence. If the inverse PCR reaction had correctly amplified 5' upstream sequences of the *PDF1.2* gene then these primers should amplify from genomic DNA all sequences of the *PDF1.2* gene that are 5' to position 326 on the T04323 sequence and this region should extend as far as the Sph1 site in the 5' upstream promoter region. Using 10 ng of intact genomic DNA of *Arabidopsis* ecotype Col-0 as a template, a PCR reaction was carried out with these primers as described above using 58 °C as the annealing temperature.. This PCR yielded a single fragment of approximately 1.6 kb in size as judged by gel electrophoresis. The band was excised from the gel and digested with HindIII and ligated into HindIII cut and dephosphorylated pEMBL18+. This clone was termed pFAJ3085 and the insert was sequenced completely on both strands with overlap using fluorescently labelled dideoxynucleotide terminators and an Applied Biosystems 373A DNA Sequencer.

Construction of *PDF1.2* Promoter-Reporter Constructs and Transformation of *Arabidopsis* and Tobacco

The promoter fragment and part of the coding sequence of the *PDF1.2* gene (positions 1 - 1254 in pFAJ3085 insert) was amplified using primers OWB272 [5' - GAGAGAGGATCCTGATGGAAGCAAACTTAGCCATG - 3', BamHI site underlined] and OWB273. This reaction used 1 ng of pFAJ3085 as template and was carried out as before using an annealing temperature of 56 °C. The predicted fragment size was obtained and gel purified. This fragment was digested with both HindIII and BamHI and then ligated into the commercial binary vector plasmid pBI101.3 (Clontech Inc.). This vector contains a T-DNA region that can be transferred to plants using *Agrobacterium tumefaciens* as an intermediary. On the T-DNA is a selectable marker gene conferring kanamycin resistance when expressed in plant cells. In addition, there is a polylinker containing HindIII and BamHI sites situated immediately 5' of the coding region of the *UidA* cistron from *Escherichia coli* (encoding the enzyme β-glucuronidase or GUS), followed by the terminator of the nopaline synthase gene from *Agrobacterium tumefaciens* (tNOS). Insertion of the HindIII/BamHI digested promotor of the *PDF1.2* gene amplified by OWB272 and OWB273 (pPDF1.2) into this region leads to a gene fusion where one would predict that the initiation of translation at the predicted normal translation initiation codon of the *PDF1.2* gene (positions 1233-1235 in pFAJ3085) would lead to production of a GUS fusion protein with the following N-terminal sequence: MAKFASIRIPGYGQSL*M*, where underlined amino acid residues are derived from the first seven N-terminal *PDF1.2* codons, normal font indicates amino acid residues encoded on the linker region of the pBI101.3 vector and the final italicised M residue is the N-terminal residue of the GUS enzyme encoded by the *UidA* cistron. This vector containing the chimeric pPDF1.2-GUS-tNOS gene was termed pFAJ3086. The insert in pFAJ3086 was reamplified using OWB273 and a commercial primer [GUS Sequencing Primer, Clontech Inc. 5' - TCACGGGTTGGGGTTTCTAC - 3'] and the terminal sequences of the PCR product directly sequenced to verify that the sequence of the *PDF1.2* gene had been correctly incorporated.

Plasmid pFAJ3086 was then transferred to the *Agrobacterium tumefaciens* strain LBA4404 by triparental mating using an *E. coli* HB101 strain containing the vector pRK2013 to promote conjugation (Ditta *et al.*). The *A. tumefaciens* strain containing the pFAJ3086 vector was used to transform leaf explants of *Nicotiana tabacum* cv. Xanthi - nc

by the leaf disc method (Horsch *et al.*) and to *Arabidopsis thaliana* Ecotype C24 using root explants (Valvekens *et al.*).

Disease Assays

The disease assay for *Botrytis cinerea* on *Arabidopsis* was performed as follows. *Arabidopsis* plants were grown on potting compost in a growth chamber (22°C, 14 hr photoperiod at a photon flux density of 80 uE m^{-2} s^{-1}, 70% relative humidity). Three weeks after sowing, all expanded leaves were wounded by pricking (3 pricks per leaf) with a needle. The wound sites were covered with a 5µl droplet of a suspension of *Botrytis cinerea* (10^5 spores/ml) in half strength potato dextrose broth (Difco). The inoculated plants were placed randomly in a propagator flat with a clear polystyrene lid and incubated as above except that the photon flux density was reduced to 50 µE m^{-2} s^{-1}. After 2 days, the lids were taken off and the plants further incubated under the same conditions. Plants were considered dead when the stem including the shoot apex and the youngest leaves were completely decayed.

Susceptibility of *Arabidopsis* to *Peronospora parasitica* was tested as follows. Conidia of *P. parasitica* pathovar Wela were collected by gently shaking infected leaves of *Arabidopsis* ecotype Weiningen in water. The conidial suspension was adjusted to 10^5 spores/ml and sprayed on four-week-old plants using a paint spray. The inoculated plants were placed randomly in a propagator flat with a clear polystyrene lid and incubated for 7 days at 20 °C with a photoperiod of 8 h at a photon flux density of 80 µE m^{-2} s^{-1}. Disease progression was examined by staining the leaves for fungal structures using the lactophenol/cotton blue staining method (Keogh *et al.*).

EXAMPLE 1

A Plant Defensin with Antifungal Activity Accumulates in Pathogen-Stressed *Arabidopsis* Leaves

To investigate whether the pathogen-induced plant defensin in *Arabidopsis* leaves is biologically active, we attempted to purify the protein by a method previously developed for the purification of Rs-AFP3 and Rs-AFP4 from fungus-infected radish leaves (Terras *et al.*, 1995). In short, the purification method consists of passage of a crude leaf protein extract over an anion exchange column at pH 7.5, passage of the unbound proteins over a cation

exchange column at pH 5.5, elution of the bound proteins at high ionic strength and, finally, separation of the eluted proteins over a reversed-phase chromatography column. Comparison of the reversed-phase chromatogram of proteins from leaves of non-inoculated *Arabidopsis* plants with that of proteins from leaves inoculated with *A. brassicicola*, led to the identification of a peak present only in the latter (peak 1 in Figure 3A). SDS-PAGE analysis showed that the protein in this peak fraction runs as a single 5 kDa band comigrating with Rs-AFP1 (Figure 3B and 3C). This protein was equally well recognized as Rs-AFP1 in immunoblots prepared from SDS-PAGE gels and developed with anti-Rs-AFP1 antiserum (Figure 3C). Furthermore, the protein in the peak 1 fraction was demonstrated to inhibit the growth in vitro of *A. brassicicola* (Figure 3D) as well as that of *Fusarium culmorum* (results not shown). The growth inhibition was characterized by hyphal hyperbranching and tip swelling as observed for Rs-AFP1 (Figure 3D) and related plant defensins from other Brassicaceae, including PDF1.1 (previously called At-AFP1) from *Arabidopsis* seed (Terras *et al.*, 1993).

EXAMPLE 2

Systemic Induction of *Arabidopsis* Plant Defensins upon Pathogen Stress

We have previously shown that plant defensin genes in radish leaves are systemically induced upon local infection with *A. brassicicola* (Terras *et al.*, 1995). To investigate whether this is also the case in *Arabidopsis*, we analyzed the induction of plant defensins in *Arabidopsis* leaves infected with *A. brassicicola* and non-treated leaves of infected plants (systemic leaves), both by ELISA (using antigen affinity-purified anti-Rs-AFP1 antiserum) and by RNA gel blot analysis (using *PDF1.2* as a probe for defensins). At the protein level, plant defensins were found to increase from amounts below the detection limit (0.05 µg/mg total soluble protein) at the sampling time just before inoculation to up to 10 and 3 µg/mg total soluble protein in the pathogen-treated leaves and non-treated, systemic leaves at 72h after inoculation, respectively (Figure 4A). Steady state levels of defensin mRNA were raised following inoculation both in pathogen-treated and non-treated, systemic leaves and the mRNA amounts in both of these leaf samples reached a maximum at 48h after inoculation (Figure 4B).

EXAMPLE 3

Induction of *Arabidopsis* Plant Defensins upon Chemical Treatments

Most thus far studied genes that are induced systemically upon pathogen stress can be induced at least partially by salicylic acid (SA) or 2,6-dichloroisonicotinic acid (INA), a synthetic compound mimicking the action of SA (Ward *et al.*, 1991). In *Arabidopsis*, it has been shown that PR-1, PR-2 and PR-3 are strongly induced by both SA and INA treatment (Uknes *et al.*). However, no induction of plant defensin gene expression could be observed upon SA or INA treatment, whether assessed by RNA blot analysis or by ELISA (Figure 5). In contrast, application of ethylene or methyl jasmonate significantly increased the level of plant defensin gene expression. No increase in plant defensin gene expression was detected in the appropriate control treatments i.e. incubation in air (control for ethylene) or application of 0.1% (v/v) ethanol (solvent control for methyl jasmonate). The expression of PR-protein genes (PR-1, PR-2, PR-3) in *Arabidopsis* has previously been shown not to respond to ethylene treatment (Lawton et al. 1994). Taken together, these data indicate that plant defensin genes and PR-protein genes are induced by different groups of chemicals.

The chemicals paraquat and rose bengal were also tested for their effect on expression of plant defensin genes. Treatment of plants with paraquat or rose bengal is known to result in the formation of the reactive oxygen species superoxide anion and singlet oxygen, respectively (Bowler *et al.*, 1992; Green and Fluhr, 1995), and hence to cause oxidative stress. Plant defensins protein and mRNA were strongly induced by either paraquat or rose bengal (Figure 5).

Wounding of *Arabidopsis* leaves did not result in accumulation of detectable levels of plant defensin mRNA when assessed 48h after treatment (Figure 5). Because a number of wound-induced genes are known to be switched on transiently at relatively short time periods after treatment (Berger *et al.*, 1995; Warner *et al.*, 1993) we also verified plant defensin gene expression, both by RNA blot analysis and ELISA, at 3, 6, 9, 12, 24 and 48h after wounding. However, expression of plant defensin genes could not be detected at any of the analyzed time points, irrespective of whether wounding was applied by making incisions in the leaves with a scalpel or by crushing the leaves with forceps (results not shown).

EXAMPLE 4

Systemic Induction of Plant Defensins in *Arabidopsis* Follows a Jasmonate and/or ethylene-dependent pathway

Because SA and INA were unable to induce the synthesis of plant defensins, we further investigated the role of SA in the induction of plant defensins following fungal infection. Therefore, we measured plant defensin gene expression in *Arabidopsis* plants known to be genetically modified in the SA-signalling pathway. The *npr1* mutant displays a blocked SA-signalling pathway as it is unable to express PR-protein genes upon SA treatment or pathogen infection (Cao *et al.*, 1994). In contrast, the *cpr1* mutant has constitutively elevated SA and PR-protein levels even in the absence of any stress signal.

Plants of different *Arabidopsis* lines affected in the SA-signalling pathway were either mock-inoculated with water or inoculated with an *A. brassicicola* spore suspension and treated and non-treated (systemic) leaves were harvested after 72h. The expression of plant defensin genes was measured both by RNA blot analysis and ELISA. When wild-type (Col-0) plants were inoculated with *A. brassicicola*, expression of plant defensin genes was induced in both pathogen-treated leaves and non-treated, systemic leaves when compared to that of the corresponding leaves of mock-inoculated plants (Figure 6). In both the *npr1* mutant and the *cpr1* mutant plant defensin gene expression was similarly induced upon challenge with *A. brassicicola* and no constitutive expression was observed in a mock-inoclated *cpr1* plants.

EXAMPLE 5

The Pathway for Induction of Plant Defensins Requires Components of the Ethylene and Jasmonate Response Pathways

As shown above, plant defensins accumulate in leaves of *Arabidopsis* plants after application of exogenous ethylene or methyl jasmonate. To clarify the role of these two plant hormones in the induction of plant defensins, we investigated expression of plant defensin genes in the ethylene-insensitive mutants *ein2* and *etr 1-3* (Guzman and Ecker, 1990; Chang *et al.*, 1993) as well as in the coronatine- and methyl jasmonate-insensitive mutant *coi1* (Feys *et al.*, 1994).

The induction of plant defensins was markedly affected upon fungal infection of the ethylene-insensitive or jasmonate-insensitive mutants. The induction of plant defensins

appeared to be almost totally blocked in *ein2* and *coi1* plants. The levels of plant defensins in pathogen-treated and non-treated, systemic leaves of *ein2* and *coi1* plants after inoculation with *A. brassicicola* were at least 30-fold lower than the levels in the corresponding leaf samples of similarly pathogen-treated wild-type plants. The block in pathogen-induced expression of plant defensin genes in *ein2* and *coi1* plants probably occurred at the transcriptional level since no plant defensin mRNA was detected in leaves of pathogen-treated plants. In *etr1-3* plants, expression of plant defensin genes was enhanced in the pathogen-treated leaves to levels similar to those found in pathogen-treated leaves of wild-type plants. However, in non-treated, systemic leaves of *etr1-3* plants, accumulation of plant defensins was increased to a level that was three-fold lower than in the systemic leaves of wild-type plants. This reduction was consistent throughout three independent experiments but the extent of reduction was variable.

EXAMPLE 6

Plant Defensins are Constitutively Induced in the Lesion Mimic Mutant *acd2*

The *Arabidopsis acd2* mutant spontaneously develops lesions similar to those developed by wild-type plants undergoing a hypersensitive response upon challenge with avirulent bacterial pathogens (Greenberg *et al.*, 1994). Since this mutant has previously been shown to accumulate high levels of PR-protein gene transcripts in both asymptomatic and necrotic leaves, (Greenberg *et al.*, 1994), it was considered worthwhile to assess plant defensin gene expression in *acd2* plants. Healthy asymptomatic upper rosette leaves and lower rosette leaves displaying necrotic lesions were harvested separately from 5-week-old *acd2* plants, as well as healthy upper and lower rosette leaves from wild-type (Col-0) plants grown under the same conditions. As measured by ELISA, *acd2* plants accumulated very high levels of plant defensins, estimated to constitute about 5% and 10% of total soluble proteins in asymptomatic leaves and leaves with necrotic lesions, respectively (Figure 8B). RNA blot analysis showed that transcript levels of plant defensins in necrotic as well as in asymptomatic leaves of *acd2* were strongly elevated compared to those in wild-type plants (Figure 8A).

EXAMPLE 7

Ethylene and jasmonic acid activate the *PDF1.2* gene via parallel signalling paths.

The observation that pathogen-induced expression of *PDF1.2* is blocked both by a mutant affected in the ethylene response (*ein2*) as well as by a mutant affected in the jasmonate response (*coi1*) implies that ethylene and jasmonate somehow interact with each other to effect expression of this gene. Conceptually, three different models for the interaction between ethylene and jasmonate can be conceived (Figure 10).

A first model implies that pathogen recognition leads to increased ethylene production which in turn would result in stimulated jasmonate production and subsequent *PDF1.2* activation. A second model would be identical to the first, except that the hierarchy between the ethylene and jasmonate signals would be reversed. A third model, finally, supposes that ethylene and jasmonate do not act in a sequential manner but rather via parallel pathways which both need to be activated for induction of the *PDF1.2* gene upon pathogen recognition.

It was previously shown that inoculation of *Arabidopsis* wild type plants (Col-O) with *Alternaria brassicicola* results in an increase in the levels of jasmonic acid in the leaves (Penninckx *et al.* 1996). The first model predicts that such a rise in jasmonate level upon pathogen infection would not occur in the ethylene-insensitive mutant *ein2*, whereas according to models 2 and 3, the *ein2* mutation would not affect pathogen-stimulated jasmonate production. To test these divergent predictions, *ein2* and wild type plants (Col-O) were infected with the pathogen *A. brassicicola* whereafter jasmonate levels were monitored at different time points. As shown in Figure 11, jasmonate levels in fungus-inoculated wild type plants started to rise as from 24 h after inoculation and more dramatically as from 48 h after inoculation to reach a peak level at about 72 h postinoculation. In the *coi1* mutant, pathogen-stimulated production was clearly not abolished but even more pronounced relative to the wild type plants. Hence, these results are in conflict with the predictions made based on model 1.

To further discriminate between models 2 and 3, ethylene production by wild type plants and *coi1* mutants was measured in response to inoculation with *A. brassicicola*. Model 2 predicts that the *coi1* mutant would be blocked in its ability to stimulate ethylene production upon pathogen attack, while model 3 implies that the ethylene response in the *coi1* mutants would not be reduced versus that of wild type plants. Inoculation of wild type

plants with *A. brassicicola* resulted in ethylene production levels that were about twice those in mock-inoculated plants, with a peak level reached at about 60 h after inoculation (Figure 12). A similar two-fold increase in ethylene production levels was also observed in the *coi1* mutant plants treated with *A. brassicicola* (Figure 12). The ethylene production levels in both inoculated and mock-inoculated *coi1* mutants were on average about two-fold higher relative to those in wild type plants. As the pathogen-stimulated ethylene production was clearly not abolished in the *coi1* mutants, it was concluded that model 2 is not valid. Model 3, proposing parallel ethylene and jasmonate signalling paths, is hence the only model that is in agreement with all observations.

An alternative way to verify the validity of model 1 is to treat wild type plants and *ein2* mutants with methyl jasmonate and subsequently measure plant defensin levels by ELISA two days after the treatment. Treatment of wild type plants with 50μM methyl jasmonate resulted in a plant defensin level that was at least 100-fold higher relative to that in solvent-treated wild type plants. In contrast, the plant defensin content of *ein2* mutants treated with methyl jasmonate was not elevated relative to that in solvent-treated plants (Figure 13). This observation is in conflict with model 1 which predicts that jasmonate-induced PDF1.2 accumulation would not be affected by the *ein2* mutation.

The fact that treatment with methyl jasmonate alone is able to activate the *PDF1.2* gene is not necessarily in conflict with model 3 as it is well established that treatment of plants by external application of methyl jasmonate results in enhanced production of ethylene (Czapski and Saniewski, 1992). Treatment of non-sterile soil-grown plants with ethylene alone results in enhanced plant defensin accumulation (see Example 3), which is also in apparent disagreement with model 3. However, when sterile plants grown on an agar medium were treated with ethylene alone (25 ppm), no induced PDF1.2 accumulation was observed (results not shown). Treatment of sterile plants with methyl jasmonate still induced PDF1.2 accumulation, most probably because of simultaneous stimulation of ethylene production. The observation that ethylene stimulates PDF1.2 accumulation in non-sterile plants but not in sterile plants might be explained by assuming that contact of plants with micro-organisms in the soil alters their responsiveness to ethylene treatment.

It is to be noted that whilst model 3 is considered to be the most likely model, it may be shown with further experimentation that one of the other models is more likely to be the model which is best describes the interaction between ethylene and jasmonate.

EXAMPLE 8

Development of a scorable marker gene for the induction of the jasmonate and/or ethylene-dependent defence pathways in *Arabidopsis* and tobacco.

The *Arabidopsis PDF1.2* gene promoter was cloned via an inverse PCR strategy using primers based on the *PDF1.2* cDNA sequence (genbank accession number T04323). This procedure resulted in the cloning of a 1616 bp genomic DNA fragment whose sequence is shown in Figure 14. The sequence of this genomic fragment at positions 1202 - 1296 and 1388 - 1616 matched exactly the sequence of the *PDF1.2* cDNA from positions 1 - 326. The interuption in the match of the genomic sequence at positions 1297 - 1387 relative to the cDNA sequence is presumed to represent a 91 bp intron which is situated within the coding sequence for the PDF1.2 signal peptide. The sequences surrounding the predicted intron splice sites were consistent with consensus sequences observed in other plant genes. Importantly, the genomic fragment contained 1201 bp 5' of the cDNA sequence and 1232 bp upstream of the predicted translational start of the PDF1.2 gene product. Experiments were then designed to test whether the DNA sequence 5' of the translational start codon of the *PDF1.2* gene might contain a promoter with regulatory elements that determine pathogen-induced local and systemic expression of this gene as well as induction by jasmonates and other chemical stimulants that will induce accumulation of the PDF1.2 gene product. To this end, the first 1254 bp of the genomic fragment encompassing the putative promoter elements together with the 5' untranslated leader and a part of the region encoding the first seven *PDF1.2* codons was linked as a translational fusion to the coding region of the *Escherichia coli UidA* gene (encoding β-glucuronidase or GUS) which in turn was hooked up to the *Agrobacterium tumefaciens* nopaline synthase gene terminator (tNOS) . The resulting pPDF1.2-GUS-tNOS expression cassette was transferred within a plant transformation vector into *Arabidopsis thaliana* ecotype C24 by *Agrobacterium tumefaciens* - based root transformation.

The ability of the pPDF1.2-GUS-tNOS transgene to respond to jasmonic acid treatment was first assessed on the progeny of six independent T0 transgenic *Arabidopsis* lines. Seeds of each of these lines were germinated aseptically, either on Murashige and Skoog agar medium containing 1 % sucrose and 50 μM kanamycin or the same medium supplemented with 10 μM jasmonic acid. Seedlings were harvested 10 days after germination and their β-glucuronidase activity measured fluorometrically using 4-methylumbelliferyl glucuronide as a substrate according to the procedure of Jefferson (1987). The β-glucuronidase activity was normalized to the total protein content in each sample, as determined by the method of Bradford (1976) using bovine serum albumin as a standard.

As shown in Table 1, all lines tested showed a marked increase (ranging from 9 to 25-fold) of the β-glucuronidase activity in the jasmonate treated seedlings versus the untreated seedlings. Line 19, which showed the strongest relative jasmonate-induced increase of β-glucuronidase activity was further bred by self-pollination to obtain T3 generation seed that was homozygous for the transgene. This line was used in all subsequent analyses.

Table 1 β-glucuronidase activity in 10-day-old seedlings of six independent *Arabidopsis* lines carrying the pPDF1.2-GUS-tNOS transgene after germination in the absence or the presence of 10 μM jasmonic acid.

Transgenic line	β-glucuronidase activity *		Fold induction
	control	JA-treated	
3	115	2895	25.2
4	113	978	8.7
5	296	2676	9.0
6	149	2166	14.5
7	21	203	9.7
19	8	202	25.2

* expressed as pmoles 4-methylumbelliferone released from 4-methylumbelliferyl glucuronide at 37°C per min and per mg soluble protein

The *PDF1.2* gene was shown by RNA gel blot analyses and ELISA assays to be systemically induced upon inoculation of *Arabidopsis* leaves with the fungal pathogen *Alternaria brassicicola*. The expression of the pPDF1.2-GUS-tNOS reporter gene in transgenic four-week-old *Arabidopsis* plants in response to inoculation by the fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea*, was assessed by β-glucuronidase activity measurements both in inoculated and non-inoculated systemic leaves 48 h following treatment of the transgenic plants. *A. brassicicola* was inoculated by applying 5 µl drops of a conidial spore suspension (5 x 10^5 spores/ml in water) on the lower rosette leaves (4 drops per leaf). *B. cinerea* was inoculated as 5 µl drops of a 5x10^5 spores/ml conidial spore suspension in half strength potato dextrose broth (Difco) covering a needle prick wound applied on the lower rosette leaves (1 drop per wound, 4 wounds per leaf). As shown in Figure 15, β-glucuronidase activity was strongly increased following inoculation with either *A. brassicicola* or *B. cinerea* both in the inoculated leaves and the non-inoculated, systemic leaves, relative to the mock-inoculated controls. This indicates that the pPDF1.2-GUS-tNOS reporter gene is a suitable marker for systemic pathogen-induced gene expression in *Arabidopsis*.

Four-week-old pPDF1.2-GUS-tNOS transgenic *Arabidopsis* plants were treated with various chemicals to investigate their ability to induce expression of the reporter gene. The chemicals used in these tests were jasmonic acid (JA, 500 µM in 0.1 % ethanol), methyl jasmonic acid (MeJA, 50 µM in 0.1 % ethanol), salicylic acid (SA, 5 mM in H_2O), 2,6-dichloroisonicotinic acid (INA, 1 mg/ml in H_2O), 1,2,3-benzothiadiazole-7-carbothioic acid S-methylester (BTH, 300 µM in H_2O), paraquat (PQ, 25 µM in H_2O). The chemical solutions were applied on the fully expanded leaves of the plants as 5 µl drops deposited on the upper leaf surface (5 drops per leaf). A separate set of plants was also treated by exposure to ethylene (25 ppm) in an air-tight chamber. Following treatment for 48 hours with either the chemicals or the appropriate controls, leaves from treated plants were harvested and β-glucuronidase activity measured.

As shown in Figure 16, treatment with either JA, MeJA, or paraquat induced the pPDF1.2-GUS-tNOS reporter gene, whereas applications of either SA, INA, BTH or ethylene were without effect. These findings corroborate the expression studies based on RNA gel blot analyses and ELISA assays of the *PDF1.2* gene in response to chemical

treatments, except for ethylene treatment. Although ethylene treatment was found to increase PDF1.2 levels in non-sterile soil-grown plants (see Example 3), this gaseous hormone did not boost PDF1.2 accumulation in sterile plants (see Example 7). Hence, non-sterile culture conditions appear to increase the responsiveness of *Arabidopsis* plants to ethylene. This increased responsiveness may be sufficient to activate the endogenous *PDF1.2* gene but not the pPDF1.2-GUS-tNOS chimeric reporter gene.

To verify whether SA, INA and BTH were applied under conditions that would induce SA-dependent pathogen-induced genes, the above tests were also performed on transgenic *Arabidopsis* plants carrying a chimeric transgene consisting of the β-glucuronidase coding region under the control of the promoter of the *Arabidopsis* β-1,3-glucanase PR-2 type gene, named *Bgl2* (Bowling *et al.*, 1994, Plant Cell 6, 1485-1857). This chimeric gene is hereunder abbreviated as pBgl2-GUS-tNOS. As shown in Figure 3, treatment of transgenic pBgl2-GUS-tNOS *Arabidopsis* plants with SA and its functional analogs INA and BTH did result in markedly increased β-glucuronidase activity, whereas neither JA, MeJA, ethylene nor paraquat had any effect. It has previously been shown that SA, INA and BTH induce SA-dependent pathogen-inducible genes in *Arabidopsis* such as *PR-1*, *PR-2* and *PR-5* (Uknes *et al.*, 1992).

Taken together, these data indicate that the pPDF1.2-GUS-tNOS reporter gene responds systemically to pathogen infection without the intervenence of SA, indicating that it is an appropriate scorable marker for SA-independent pathogen-induced gene activation and possibly for SA-independent induced resistance.

In order to assess whether the pPDF1.2-GUS-tNOS promoter could be used as a marker for SA-independent pathogen-induced gene expression in a plant other than *Arabidopsis*, the pPDF1.2-GUS-tNOS gene was also introduced into the tobacco cultivar Xanthi-nc by *Agrobacterium*-mediated leaf disc transformation. T2 generation plants were selected that were homozygous for the transgene. Systemic pathogen-induced expression in a transgenic tobacco line was assessed on 8-week-old plants after inoculation with tobacco mosaic virus of the leaf just below the youngest fully expanded leaf. Tobacco cultivar Xanthi-nc is resistant to tobacco mosaic virus and reacts to the virus by producing a hypersensitive response, thus preventing the virus from spreading beyond the lesions. The youngest fully expanded leaf and the leaf above the youngest fully expanded leaf were

harvested separately at 2, 4 and 6 days after inoculation and the β-glucuronidase activity was measured. Tobacco mosaic virus inoculum was prepared by grounding a leaf of tobacco cultivar Hicks preinfected with tobacco mosaic virus in 50 mM sodium phosphate buffer (pH7) at 1 g tissue per 10 ml buffer. The suspension was diluted 10-fold in buffer and applied on tobacco leaves by rubbing with carborundum powder. Control plants were mock inoculated with buffer and powder only. As shown in Figure 17, β-glucuronidase activity was markedly increased in the inoculated leaves as from two days after inoculation and in the systemic leaves as from four days after inoculation. In addition, the inducibility of the reporter gene was assessed by treating leaves with salicylic acid (SA, 5 mM in H_2O), methyl jasmonic acid (MeJA, 50 μM in 0.1 % ethanol) paraquat (PQ, 25 μM in H_2O) and by wounding. The chemicals were applied as four droplets of 100μl on the fully expanded leaf of 8-week-old plants. Wounding was tested by crushing leaves at 2 cm intervals with forceps with serrated ends. The β-glucuronidase content in the treated leaves and appropriate controls was measured 48 h after treatment.

As shown in Figure 18, both methyl jasmonate and paraquat caused a more than 35-fold increase in β-glucuronidase activity. In contrast, wounding and salicylic acid treatment led only to a 2-fold increase in activity. Verification of the activation of the tobacco PR-1 gene by RNA gel blot analysis in the tobacco plants treated in the same way indicated that, like in the case of *Arabidopsis*, this gene was induced only by salicylic acid but not by methyl jasmonate, paraquat or wounding (not shown).

These results indicate that the *PDF1.2* promoter is activated in a SA-independent way in tobacco, a plant from the family Solanaceae, while it can be induced systemically throughout the plant upon pathogen challenge. Hence, the pPDF1.2-GUS-tNOS reporter gene can be used in different plants for the purpose of screening compounds, microorganisms or physical treatments that induce the SA-independent defence pathway.

EXAMPLE 9

Synergistic induction of the plant defensin promoter by ethylene and jasmonate.

Our finding that jasmonate and ethylene act via parallel paths to activate the *PDF1.2* gene (see Example 7) suggests that combinations of jasmonate and ethylene may have a synergistic effect on the activation of this gene. To test this hypothesis, ethylene and methyl

jasmonate were combined at doses whereby each of both compounds alone could not activate the pPDF1.2-GUS-tNOS gene in the transgenic *Arabidopsis* reporter plants. As shown in Figure 19, treatment of *Arabidopsis* leaves with drops of 0.5μM methyl jasmonate in an atmosphere containing 25 ppm ethylene resulted in a very high β-glucuronidase activity, whereas each single treatment did not cause increased β-glucuronidase levels. A significant but lower level of synergy was detected upon combining ethephon (a compound that generates ethylene when sprayed on plants) at 333 μM and methyl jasmonate at 0.5 μM (Figure 19). In this test, ethephon was applied at a rate that was 10-fold lower than the rate normally used to activate senescence processes in plants.

These findings suggest that treatment of plants with a mixture of compounds that separately activate the ethylene response and the jasmonate response paths may result in a more effective stimulation of natural resistance against particular types of pathogens.

EXAMPLE 10

Evidence that the jasmonate and/or ethylene-dependent defence pathways plays an important role in defence of *Arabidopsis* against some pathogens.

To test the disease susceptibility of *Arabidopsis* mutants affected in the jasmonate and/or ethylene-dependent defence pathways, a disease bioassay was developed for the Ascomycetous necrotrophic fungal pathogen *Botrytis cinerea* (teleomorph = *Botryotinia fuckeliana*). This assay consisted of applying drops of a *Botrytis cinerea* spore suspension over needle prick wounds, whereafter succumbing of inoculated plants was monitored. Figure 20 shows the number of deceased plants in tests set up with series of wild type *Arabidopsis* plants (Col-0), ethylene insensitive mutants (*ein2*), and jasmonate insensitive mutants (*coi1*). On average, 41% of all inoculated *ein2* mutants and 86 % of all inoculated *coi1* mutants succumbed after 16 days, versus only 1% of the wild type plants (Figure 20). Decay of the *ein2* and *coi1* plants was followed by abundant formation of conidia. Preliminary tests indicated that *npr1*, a mutant affected in the salicylate-dependent defence response, was as resistant to *Botrytis cinerea* infection as the Col-O wild type plants. These results point to the importance of the *jasmonate and/or ethylene*-dependent defence response pathways for establishing resistance of *Arabidopsis* plants against the grey mould fungus *Botrytis cinerea*.

Wild type plants (Col-0) and the mutants *npr1*, *ein2*, and *coi1* were also subjected to a disease bioassay performed with the Oomycetous biotrophic fungal pathogen *Peronospora parasitica* pathovar Wela. The infection by this fungus was assessed by detection of fungal structures in inoculated leaves using the lactophenol/trypan blue staining method. These tests showed that Col-O, *ein2* and *coi1* did not support any detectable growth of the pathogen whereas the *npr1* mutant was heavily infected by fungal hyphae that abundantly formed oospores (Figure 21).

Taken together these data indicate that the *jasmonate and/or ethylene*-dependent defence pathways described here is a key determinant in succesful defence against infection attempts by *Botrytis cinerea* but not by *Peronospora parasitica*, and vice versa for the salicylate-dependent defence pathway. Hence, at least two distinct defence pathways that are effective against separate classes of pathogens appear to operate in plants. Importantly, this finding implicates that a broader spectrum of induced resistance will be reached upon treatment of plants with mixtures of compounds that induce both the *jasmonate and/or ethylene*-dependent defence pathways and the salicylate-dependent defence pathway.

If the jasmonate and/or ethylene-dependent pathogen-inducible defence response plays a pivotal role in host defence against some pathogens, as deduced from the enhanced disease susceptibility phenotype observed for ethylene insensitive and jasmonate insensitive *Arabidopsis* mutants, then it can be expected that pretreatment of plants with compounds activating this response would result in reduced susceptibility to certain pathogens.

Against at least *B. cinerea* we have shown that at least *Arabidopsis* plants can mount resistance by activating *jasmonate and/or ethylene*-dependent genes and not salicylate-dependent genes, which may explain the lack of protection conferred by 1,2,3-benzothiadiazole-7-carbothioic acid S-methylester against this pathogen (Lawton *et al.*).

Nearly all crop plants are susceptible to a range of pathogens rather than to one or two pathogenic microorganisms. It is therefore important that chemicals used for disease control confer resistance to a spectrum of pathogens that is as broad as possible. We have described here previously unidentified defence pathways, called the *jasmonate and/or ethylene*-dependent defence pathways, that not only differs genetically from the well-studied salicylate-dependent defence pathway but also contributes to the resistance against particular pathogens that are otherwise not controlled via activation of the salicylate-dependent defence

pathway. It follows from these observations that a broader disease control spectrum can be obtained by treating plants with cocktails and compounds that activate both the salicylate-dependent defence pathway and the *jasmonate and/or ethylene* dependent defence pathways.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention. For example, the screen of the present invention may also be used for screening for chemical, biological, microbial or physical treatments of plants that result in salicylate-independent increased resistance to disease or pests.

REFERENCES

Beffa, R., Szell, M., Meuwly, P., Pay, A., Vögeli-Lange, R., Métraux, J.-P., Neuhaus, G., Meins, F., and Nagy, F. (1995). Cholera toxin elevates pathogen resistance and induces pathogenesis-related gene expression in tobacco. EMBOJ **14**, 5753-5761.

Bent, A.F., Innes, R.W., Ecker, J.R., and Staskawicz, B.J. (1992). Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. Molec. Plant-microbe Interact. **5**, 372-378.

Berger, S., Bell, E., Sadka, A., and Mullet, J.E. (1995). *Arabidopsis thaliana* Atvsp is homologous to soybean VspA and VspB, genes encoding vegetative storage protein acid phosphatases, and is regulated similarily by methyl jasmonate, wounding, sugars, light and phosphate. Plant Mol. Biol. **27**, 933-942.

Bleecker, A.B., Estelle, M.A., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241**, 1086-1089.

Bowler, C., Van Montagu, M., and Inzé, D. (1992). Superoxide dismutase and stress tolerance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43**, 83-116.

Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X. (1994). A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. Plant Cell **6**, 1845-1857.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

Brederode, F.T., Linthorst, H.J.M., and Bol, J.F. (1991). Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol. Biol. **17**, 1117-1125.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Vanderleyden, J. (1990). An automated quantitative assay for fungal growth. FEMS Microbiol. Lett. **69**, 55-60.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Osborn, R. (1995). Plant defensins : Novel antimicrobial peptides as components of the host defence system. Plant Physiol. **108**, 1353-1358.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6**, 1583-1592.

Czapski and Saniewski (1992) J. Plant. Physiol. **139**, 265-268.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). *Arabidopsis* ethylene-response gene *etr1*: Similarity of product to two-component regulators. Science **262**, 539-544.

Chen, Q.G., and Bleecker, A.B. (1995). Analysis of ethylene signal-transduction kinetics associated with seedling-growth responses and chitinase induction in wild-type and mutant *Arabidopsis*. Plant Physiol. **108**, 597-607.

Cohen, Y., Gisi, U., and Nickerman, T. (1993). Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic-methyl-ester. Phytopathol. **83**, 1054-1062.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266**, 1247-1250.

Dellaporta, S.L.J., Wood, J., Hicks, J.B. (1983) A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter **1**: 19-21

Dempsey, D.M.A., Wobbe, K.K., and Klessig, D.F. (1993). Resistance and susceptible responses of *Arabidopsis thaliana* to Turnip crinkle virus. Phytopathol. **83**, 1021-1029.

Ditta, G., Stanfield, S., Corbin, D., Helinski, D.R. (1980) Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. USA. **12**, 7347-7351.

Doares, S.H., Narvaez-Vasquez, J., Conconi, A., and Ryan, C.A. (1995). Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol. **108**, 1741-1746.

Ecker, J.R., and Davis, R.W. (1987). Plant defence genes are regulated by ethylene. Proc. Natl. Acad. Sci. USA **84**, 5202-5206.

Ecker, J.R. (1995). The ethylene signal transduction pathway in plants. Science **268**, 667-675.

Eggermont K., Goderis I.J., Broekaert W.F. (1996) High-throughput RNA extraction from plant samples based on homogenisation by reciprocal shaking in the presence of a mixture of sand and glass beads. Plant Mol. Biol. Rep. 14, 273-279.

Epple, P., Apel, K., and Bohlmann, H. (1995). An *Arabidopsis thaliana* thionin gene is inducible *via* a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol. **109**, 813-820.

Feys, B.J.F., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6**, 751-759.

Friedrich *et al.* (1996) Plant J. **10**, 61-70.

Gasch, A., Aoyana, T., Foster, R. and Chua, N-H. (1992). Gene Isolation with the polymerase Chain Reaction In Methods in *Arabidopsis* Research C. Koncz, N-H Chua, J. Schell (eds.) World Scientific Publishing Co. Ltd. Singapore pp 342-356.

Gorlach *et al.* (1996) Plant Cell, **8**, 626-643.

Green, R., and Fluhr, R. (1995). UV-B-induced PR-1 accumulation is mediated by active oxygen species. Plant Cell 7, 203-212.

Greenberg, J.T., Guo, A., Klessig, D.F., and Ausubel, F.M. (1994). Programmed cell death in plants : a pathogen triggered response activated coordinately with multiple defence functions. Cell 77, 551-563.

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2**, 513-523.

Heitz, T., Fritig, B., and Legrand, M. (1994). Local and sytemic accumulation of pathogenesis-related proteins in tobacco plants infected with tobacco mosaic virus. Molec. Plant-Microbe Interact. 7, 776-779.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants, Science **227**, 1229-1231.

Jefferson (1987) Plant Mol. Biol. Rep. **5**, 387-405.

Keogh *et al.* (1980), Trans. Br. Mycol. Soc. **74**, 329-333.

Kessmann *et al.* (1994) Annu. Rev. Phytopathol. **32**, 439-459.

Kogel *et al.* (1995). Eur. J. Plant Pathol. **101**, 319-332.

Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol. Plant-Microbe Interact. **8**, 863-870.

Lawton, K.A., Potter, S.L., Uknes, S., and Ryals, J. (1994). Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. Plant Cell **6**, 581-588.

Lawton *et al.* (1996) Plant J. **10**, 71-82.

Mauch, F., Hadwiger, L.A., and Boller, T. (1984). Ethylene: Symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitor. Plant Physiol. **76**, 607-611.

Mauch-Mani, B., and Slusarenko, A.J. (1994). Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. **7**, 378-383.

Meins, F., Neuhaus, J.M., Sperisen, C., and Ryals, J. (1991). The primary structure of plant pathogenesis-related glucanohydrolases and their genes. In Genes involved in plant defence, T. Boller, F. Meins, eds. (Springer Verlag, Berlin), pp. 245-282.

Nairn, C.J., Winsett, L., and Ferl, R.J. (1988). Nucleotide sequence of an actin gene from *Arabidopsis thaliana*. Gene **65**, 247-257.

Peña-Cortes, H., Albrecht, T., Prat, S., Weiler, E.W., and Willmitzer, L. (1993). Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta **191**, 123-128.

Penninckx *et al.* (1996) Plant Cell **8**, 2309-2323.

Potter, S., Uknes, S., Lawton, K., Winter, A.M., Chandler, D., Dimaio, J., Novitzky, R., Ward, E., and Ryals, J. (1993). Regulation of a hevein-like protein in *Arabidopsis*. Mol. Plant-Microbe Interact. **6**, 680-681.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular cloning : A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring, Harbor, NY.

Schaller, G.E., and Bleecker, A.B. (1995). Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. Science **270**, 1809-1811.

Schweizer *et al.* (1997) Plant Physiol. 114, 79-88.

Terras, F.R.G., Schoofs, H.M.E., De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F. (1992). Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J. Biol. Chem. **267**, 15301-15309.

Terras, F.R.G., Torrekens, S., Van Leuven, F., Osborn, R.W., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F. (1993). A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae species. FEBS Lett. **316**, 233-240.

Terras, F.R.G., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F. (1995). Small cysteine-rich antifungal proteins from radish: Their role in host defence. Plant Cell **7**, 573-588.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in *Arabidopsis*. Plant Cell **4**, 645-656.

Uknes, S., Winter, A., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E. and Ryals, J. (1993). Biological induction of systemic acquired resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. **6**, 692-698.

Valvekens, D., van Montagu, M. and van Lijsebettens, M., (1988) *Agrobacterium tumefaciens* mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA. **85**, 5536-5540.

Warner, S.AJ., Scott, R., and Draper, J. (1993). Isolation of an asparagus intracellular PR gene (AoPR1) wound-responsive promoter by the inverse polymerase chain reaction and its characterization in transgenic tobacco. Plant J. **3**, 191-201.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3**, 1085-1094.

White *et al.* (1979) Virology, **99**, 410-412.

CLAIMS

1. A method of protecting a plant against a pathogen, the method comprising inducing expression of a plant defensin gene by stimulating the jasmonate and/ or ethylene pathways.

2. A method according to claim 1 wherein expression of a plant defensin gene or a co-ordinately expressed gene is induced by stimulating the jasmonate and ethylene pathways.

3. A method according to claim 1 or claim 2 wherein the pathogen is necrotrophic pathogen.

4. A method according to any one of claims 1 to 3 wherein the pathogen is a microbial pathogen.

5. A method according to any one of the preceding claims wherein the pathogen is a fungus.

6. A method according to any one of the preceding claims wherein the pathways are stimulated by the application of one or more of ethylene, jasmonic acid or a jasmonate-like compound, an agent which mimics the action of ethylene, an agent which mimics the action of jasmonic acid, a non-herbicidal amount of an agent which mimics the action of ethylene, a non-herbicidal amount of an agent which mimics the action of jasmonate and an agent which causes oxidative stress.

7. A method according to any one of the preceding claims wherein stimulation of the jasmonate and/ or ethylene pathways involves the signal transduction components EIN 2 and COI 1 from *Arabidopsis*.

8. A method of inducing expression of a plant defensin gene by applying to the plant one or more of ethylene, jasmonate, an agent which mimics the action of ethylene or jasmonate and an agent which causes oxidative stress.

9. A method according to claim 7 or claim 8 wherein the agent capable of causing oxidative stress is a diphenyl herbicide such as paraquat or diquat which result in the formation of superoxide anions or rose bengal or eosine which leads to the production of singlet oxygen species.

10. A composition which is capable of inducing expression of a plant defensin gene comprising one or more of jasmonic acid, a jasmonate, ethylene, an agent which mimics the action of ethylene or jasmonate and an agent which causes oxidative stress.

11. A composition which is capable of inducing expression of a plant defensin gene comprising one or more of an ethylene-generating compound, a lipid derived signal molecule, salicylic acid, functional analogues of salicylic acid and reactive oxygen generating compounds.

12. A composition according to claim 11 wherein the ethylene-generating compound is selected from ethylene, ethephon and aminocyclopropanecarboxylic acid.

13. A composition according to claim 11 or claim 12 wherein the lipid-derived signal molecule is selected from arachidonic acid and derivatives thereof, linolenic acid and derivatives thereof and jasmonate and derivatives thereof.

14. A composition according to any one of claims 11 to 13 wherein the reactive oxygen generating compound is selected from paraquat, diquat, rose bengal and eosine.

15. A method for screening compounds for resistance-inducing activity, the method comprising applying to a plant or part of a plant a compound suspected of giving such resistance and detecting the expression of a plant defensin gene or a co-ordinately expressed gene.

16. A method according to claim 15 wherein the pathogen is a necrotrophic pathogen

17. A method according to claim 15 or claim 16 wherein the pathogen is a microbial pathogen.

18. A method according to any one of claims 15 to 17 wherein the pathogen is a fungus.

19. A method according to any one of claims 15 to 18 wherein the plant is radish, tobacco or *Arabidopsis*.

20. A method according to claim 19 wherein the plant is *Arabidopsis* and the defensin is the product of the plant defensin gene PDF 1.2 (Figure 14), a sequence that has substantial homology with the sequence of PDF 1.2 or a variant thereof.

21. A method according to any one of claims 15 to 20 wherein the compound is applied to a leaf.

22. A method according to any one of claims 15 to 21 wherein the detection is carried out by using antibodies against the gene products of PDF 1.1 or PDF 1.2.

23. A promoter which is capable of inducing the expression of a plant defensin gene comprising a region which is induced by jasmonic acid or an agent which mimics the action thereof and/ or ethylene or an agent which mimics the action thereof.

24. A promoter region comprised within the nucleic acid sequence shown in Figure 14, or a sequence that has substantial homology with that shown in Figure 14, or a variant thereof.

25. A method, a composition and a promoter substantially as hereinbefore described with reference to any one of the Figures.

Fig.1.

A

PDF1.1

```
1        AAACAACTAAGTATAAACAATAGTCATGGCTAAGTCTGCTACCATCGTTACTCTTTTCTTCGCTGCT
-29                                M  A  K  S  A  T  I  V  T  L  F  F  A  A

68       CTTGTTTTCTTTGCTGCTCTTGAAGCACCGATGGTGGTGGAAGCACAGAAGTTGTGCGAGAGGCCAAGT
-15       L  V  F  F  A  A  L  E  A  P  M  V  V  E  A  Q  K  L  C  E  R  P  S

137      GGGACATGGTCCGGAGTTTGCGGAAACAGTAACGCGTGCAAGAATCAGTGCATTAACCTTGAGAAAGCA
+9        G  T  W  S  G  V  C  G  N  S  N  A  C  K  N  Q  C  I  N  L  E  K  A

206      CGACATGGATCTTGCAACTATGTCTTCCCAGCTCACAAATGTATCTGCTACTTCCCATGTTAATCTACC
+32       R  H  G  S  C  N  Y  V  F  P  A  H  K  C  I  C  Y  F  P  C

275      AAGAGCTCTTAATGCTTAATTATAAAGTGTGTATTTCTATTAAATAAGTATGTGTCACTCTATGAGAGG

344      TCTTATGACATGTACCAGATATGTTATGTTGCTTTGTTTTAATAAGGTAAACTTTTACGG
```

Fig.1 (Cont).

B

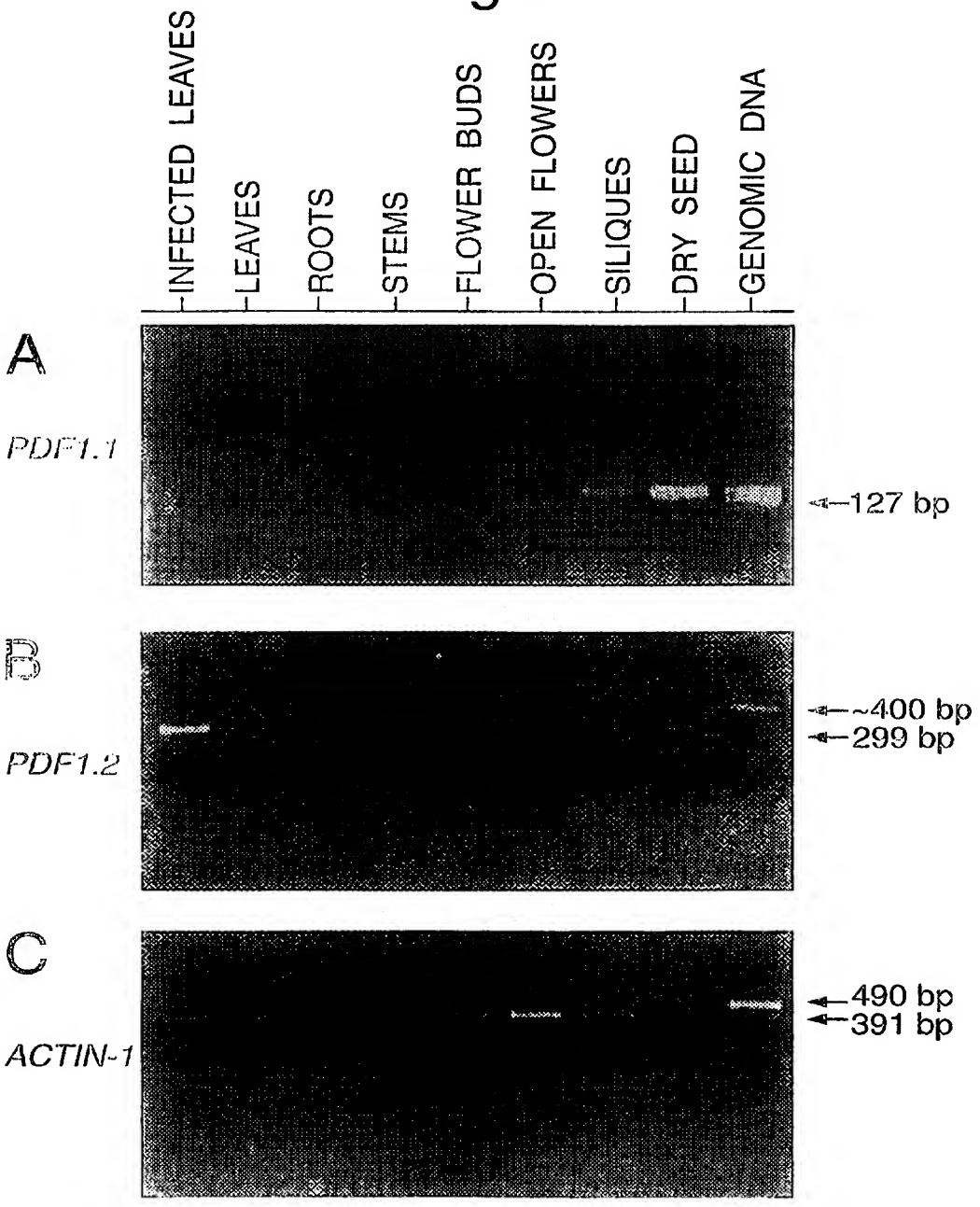
PDF1.2

```
1        ATACATTGAAAACAAAATAGTAATAATCATCATGGCTAAGTTTGCTTCCATCATCACCCTTATCTTC
-29                                      M  A  K  F  A  S  I  I  T  L  I  F

68     GCTGCTCTTGTTCTCTTTGCTGCTTTCGACGCACCGGCAATGGTGGAAGCACAGAAGTTGTGCGAGAAG
-17     A  A  L  V  L  F  A  A  F  D  A  P  A  M  V  E  A  Q  K  L  C  E  K

137    CCAAGTGGGACATGGTCAGGGGTTTGCGGAAACAGTAATGCATGCAAGAATCAGTGCATTAACCTTGAA
+7      P  S  G  T  W  S  G  V  C  G  N  S  N  A  C  K  N  Q  C  I  N  L  E

206    GGAGCCAAACATGGATCATGCAACTATGTCTTCCCAGCACACAAGTGTATCTGTTACGTCCCATGTTAA
+30     G  A  K  H  G  S  C  N  Y  V  F  P  A  H  K  C  I  C  Y  V  P  C

245    ATCTACCACTAATCTTTGGTGCTAAATCGTGTGTATTTTACATAAAAATAAGTCNCTGTCACTCTNTGA

314    GTAACTTTATGACATGCATATTTCTGGTTTTAATTGNTTATTTTNCCGCCGGTTGGT
```

C

```
PDF1.1    QKLCERPSGTWSGVCGNSNACKNQCINLEKARHGSCNYVFPAHKCICYFPC
PDF1.2    QKLCEKPSGTWSGVCGNSNACKNQCINLEGAKHGSCNYVFPAHKCICYVPC
Rs-AFP1   QKLCERPSGTWSGVCGNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC
Rs-AFP3    KLCERSSGTWSGVCGNNNACKNQCIRLEGAQHGSCNYVFPAHKCICYFPC
```

Fig.2.


INFECTED LEAVES
LEAVES
ROOTS
STEMS
FLOWER BUDS
OPEN FLOWERS
SILIQUES
DRY SEED
GENOMIC DNA
A
PDF1.1
127 bp
B
PDF1.2
~400 bp
299 bp
C
ACTIN-1
490 bp
391 bp

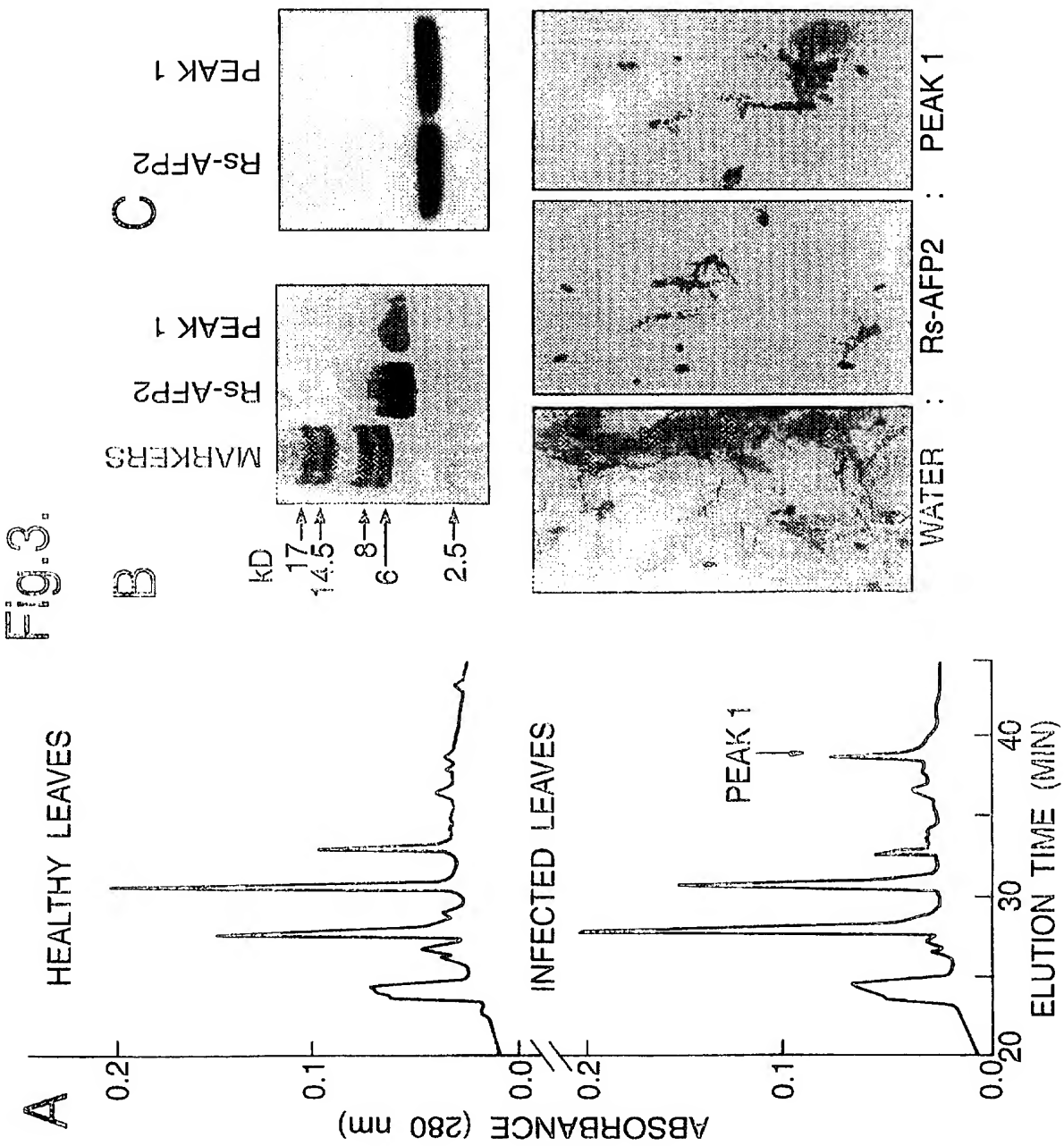

A
HEALTHY LEAVES
INFECTED LEAVES
PEAK 1
ABSORBANCE (280 nm)
0.2
0.1
0.0
0.2
0.1
0.0
20
30
40
ELUTION TIME (MIN)
B
MARKERS
Rs-AFP2
PEAK 1
kD
17
14.5
8
6
2.5
C
Rs-AFP2
PEAK 1
WATER : Rs-AFP2 : PEAK 1

Fig. 3.

Fig.4.

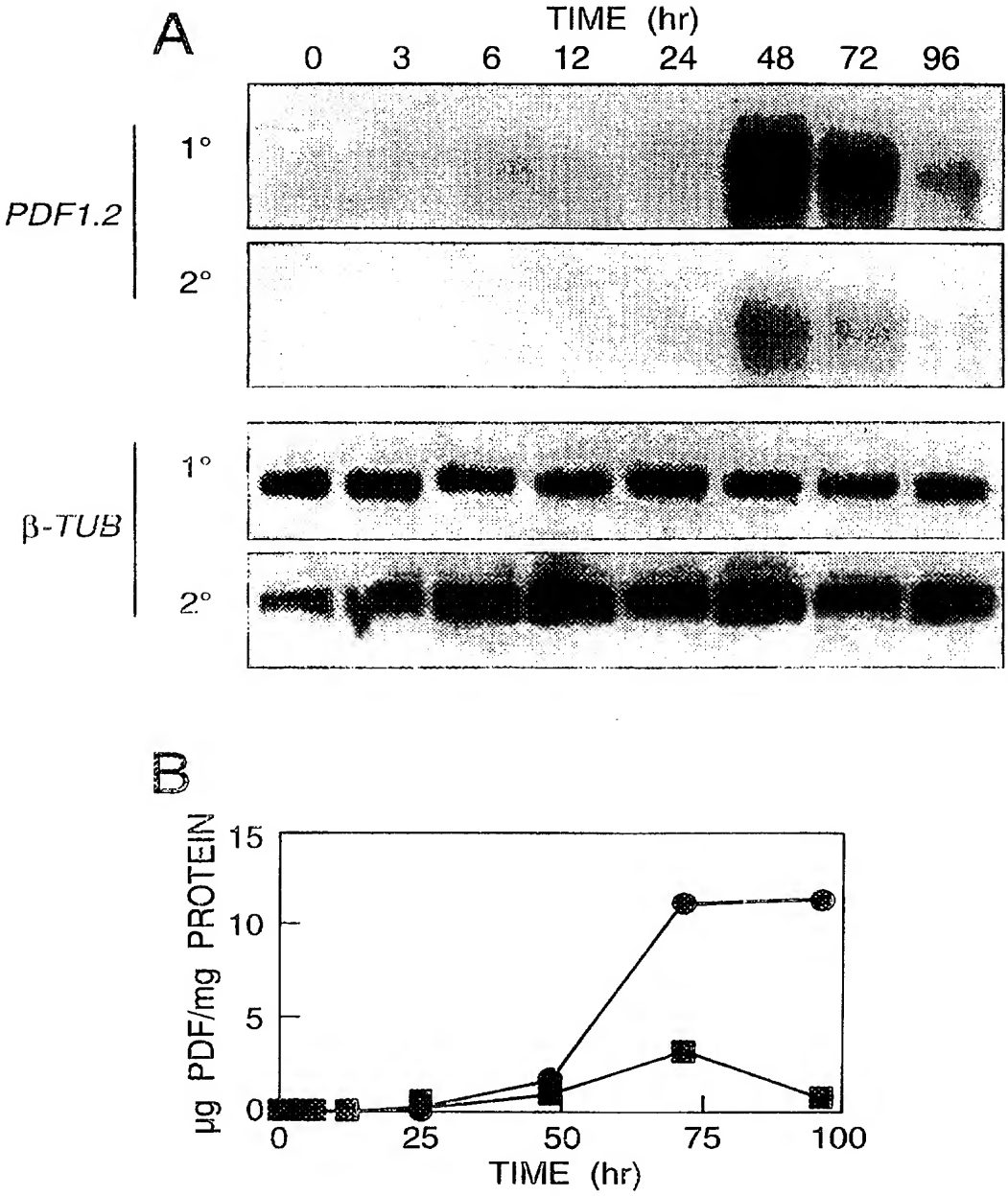

A
TIME (hr)
0 3 6 12 24 48 72 96
PDF1.2
1°
2°
β-TUB
1°
2°
B
µg PDF/mg PROTEIN
15
10
5
0
0 25 50 75 100
TIME (hr)

Fig.5.

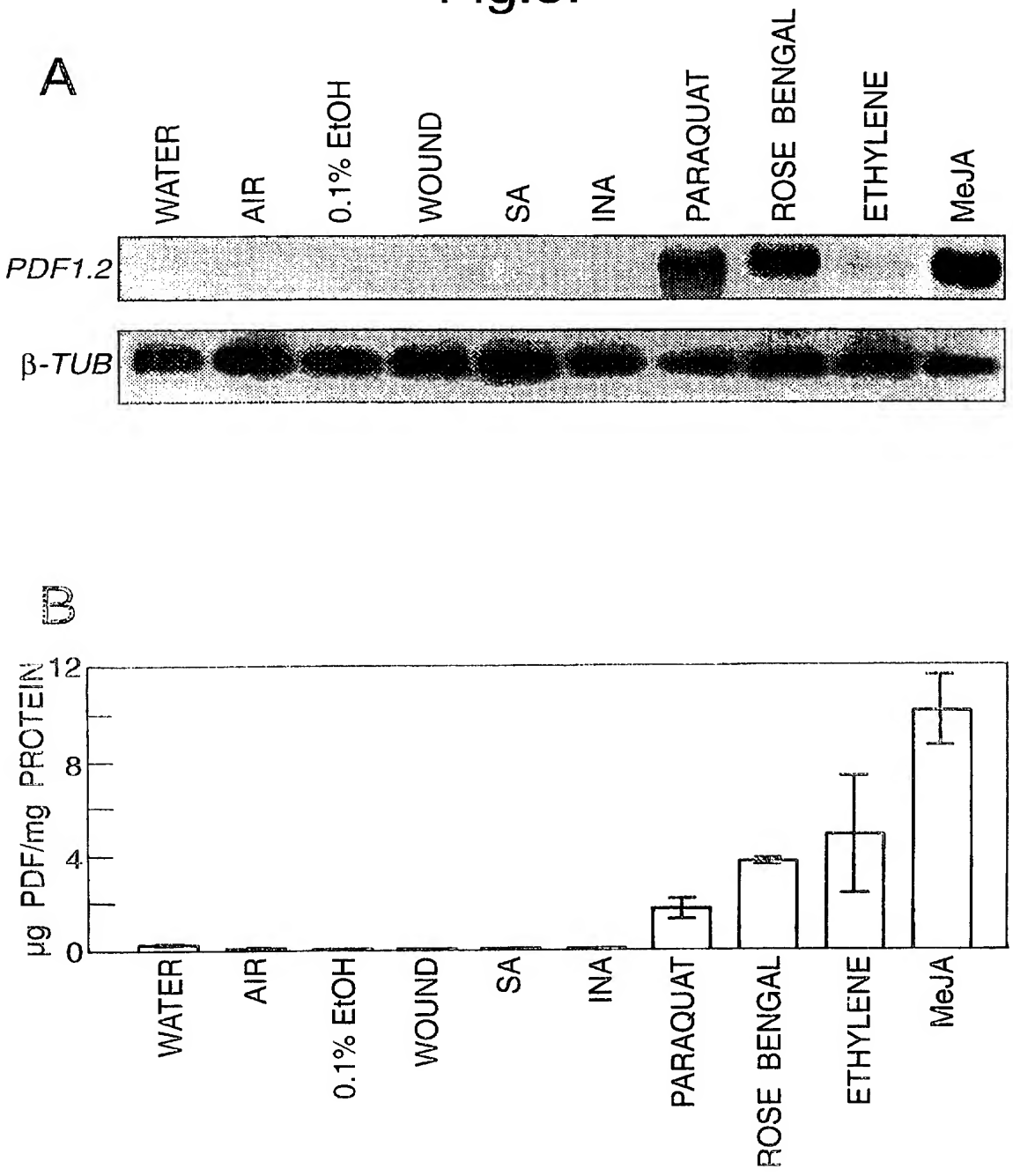

A
WATER
AIR
0.1% EtOH
WOUND
SA
INA
PARAQUAT
ROSE BENGAL
ETHYLENE
MeJA
PDF1.2
β-TUB
B
µg PDF/mg PROTEIN
12
8
4
0
WATER
AIR
0.1% EtOH
WOUND
SA
INA
PARAQUAT
ROSE BENGAL
ETHYLENE
MeJA

Fig.6.

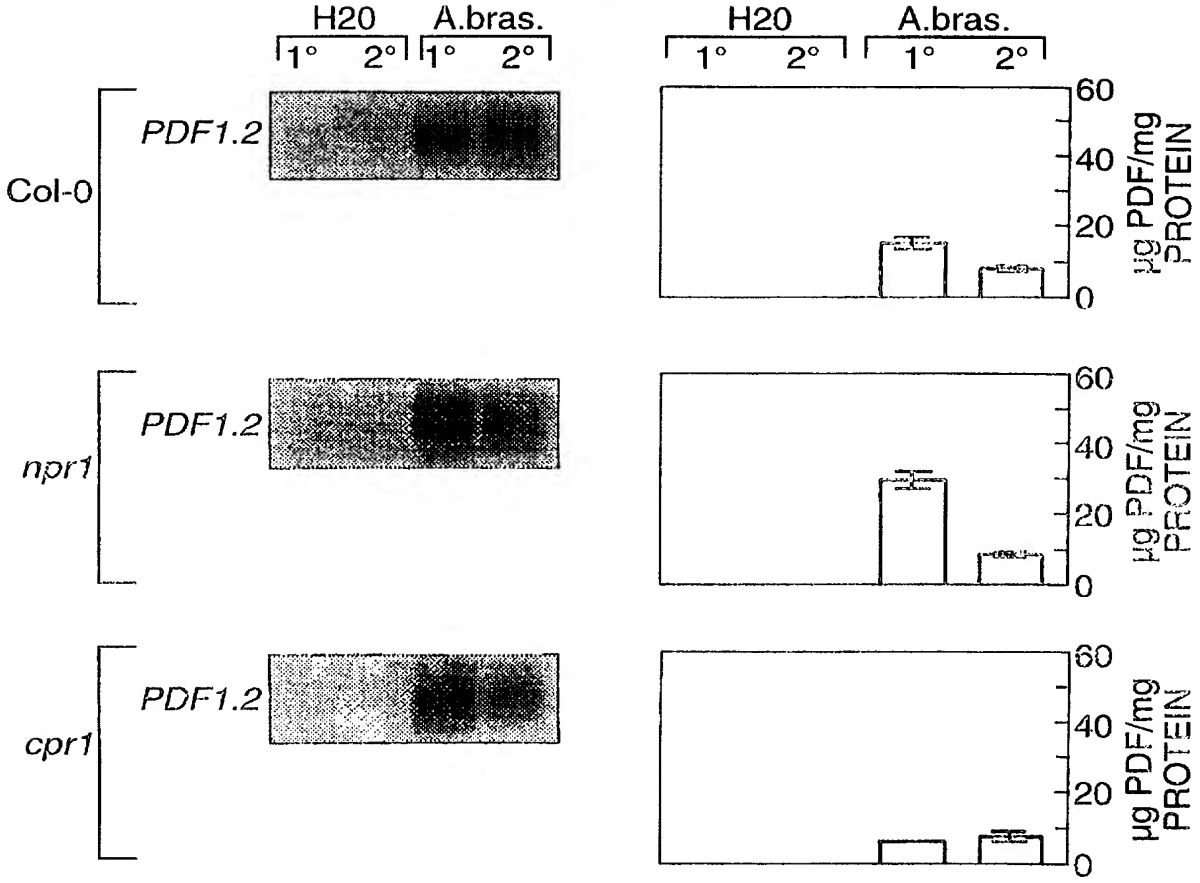

H20
A.bras.
1°
2°
1°
2°
H20
A.bras.
1°
2°
1°
2°
Col-0
PDF1.2
npr1
PDF1.2
cpr1
PDF1.2
60
40
20
0
µg PDF/mg PROTEIN

Fig.7.

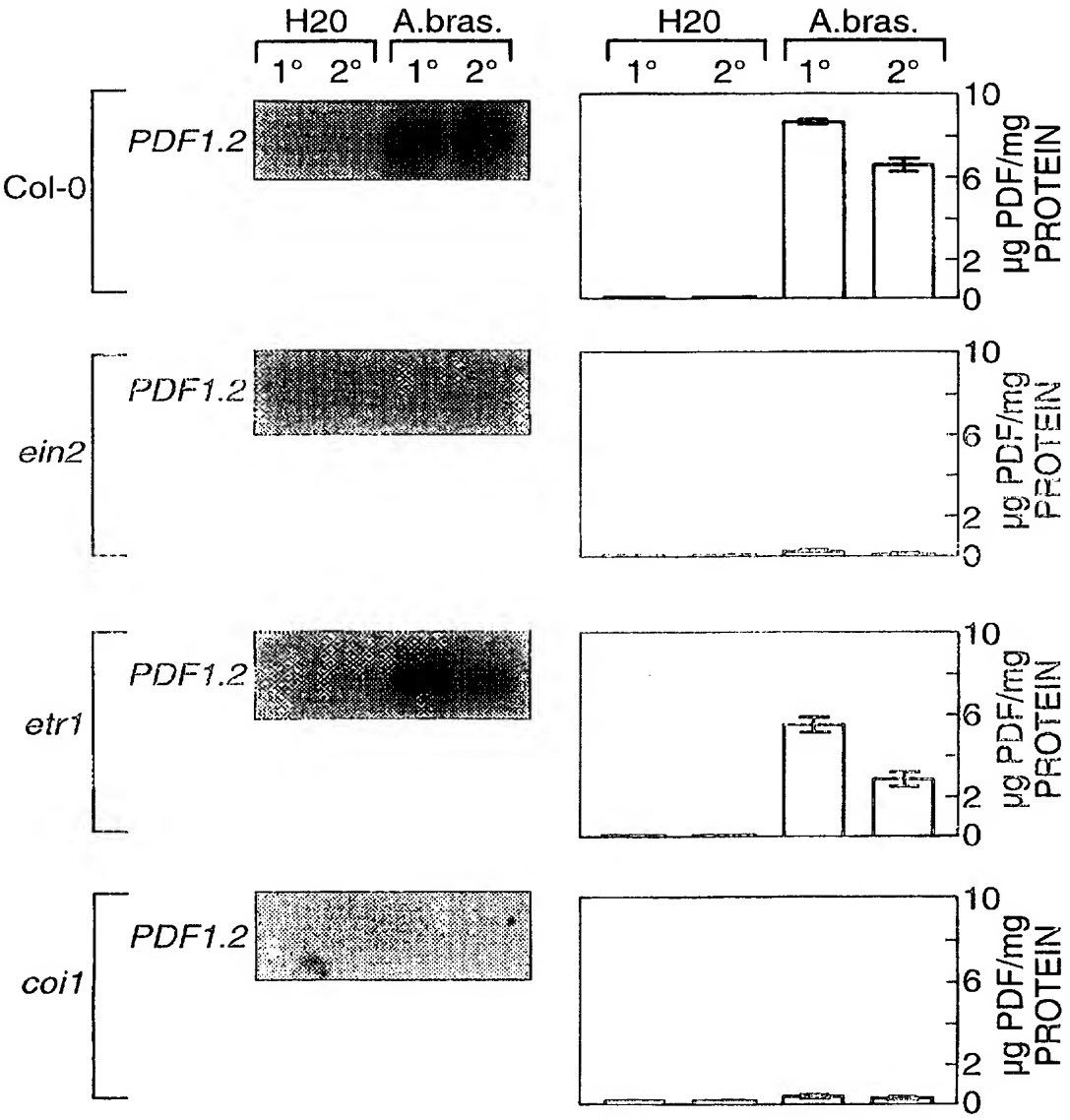

H20
A.bras.
1° 2° 1° 2°
H20
A.bras.
1° 2° 1° 2°
Col-0
PDF1.2
ein2
PDF1.2
etr1
PDF1.2
coi1
PDF1.2
10
6
2
0
µg PDF/mg PROTEIN

Fig.8.

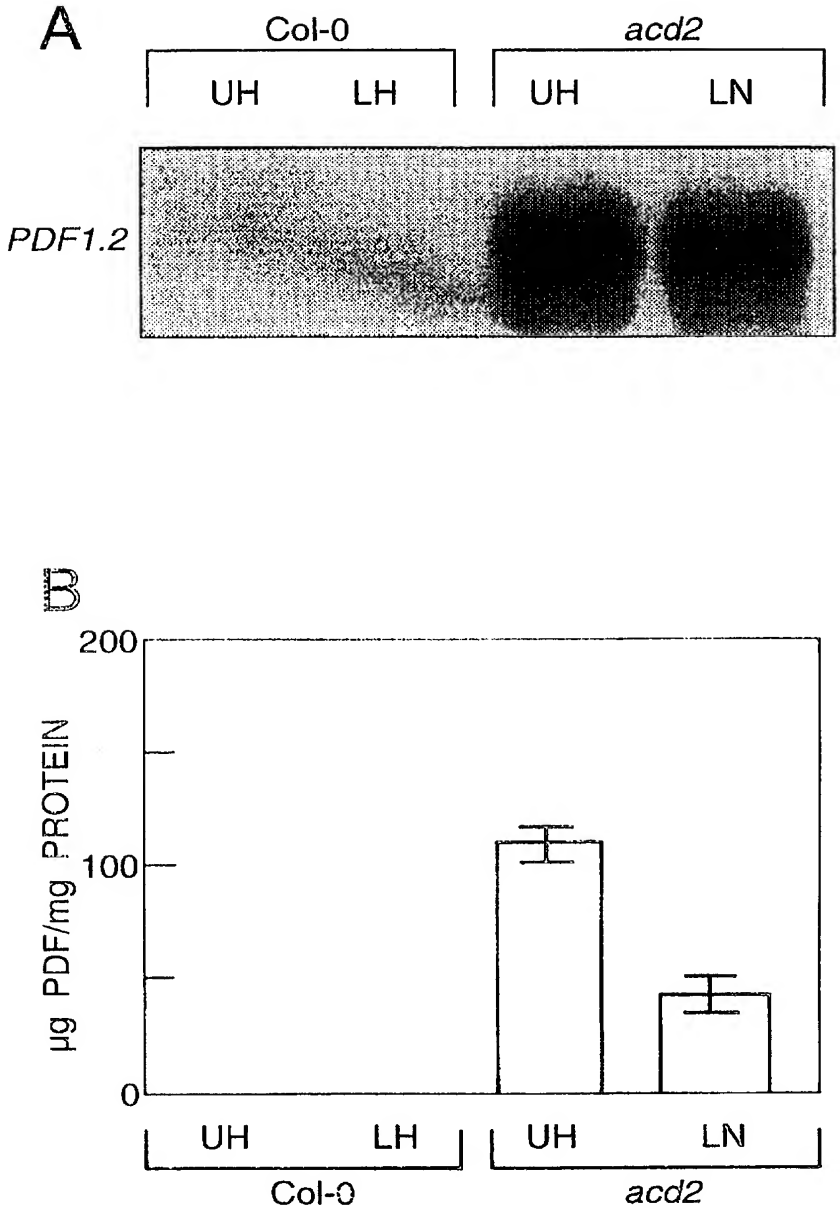

A
Col-0
acd2
UH
LH
UH
LN
PDF1.2
B
200
100
0
µg PDF/mg PROTEIN
UH
LH
UH
LN
Col-0
acd2

Fig.9.

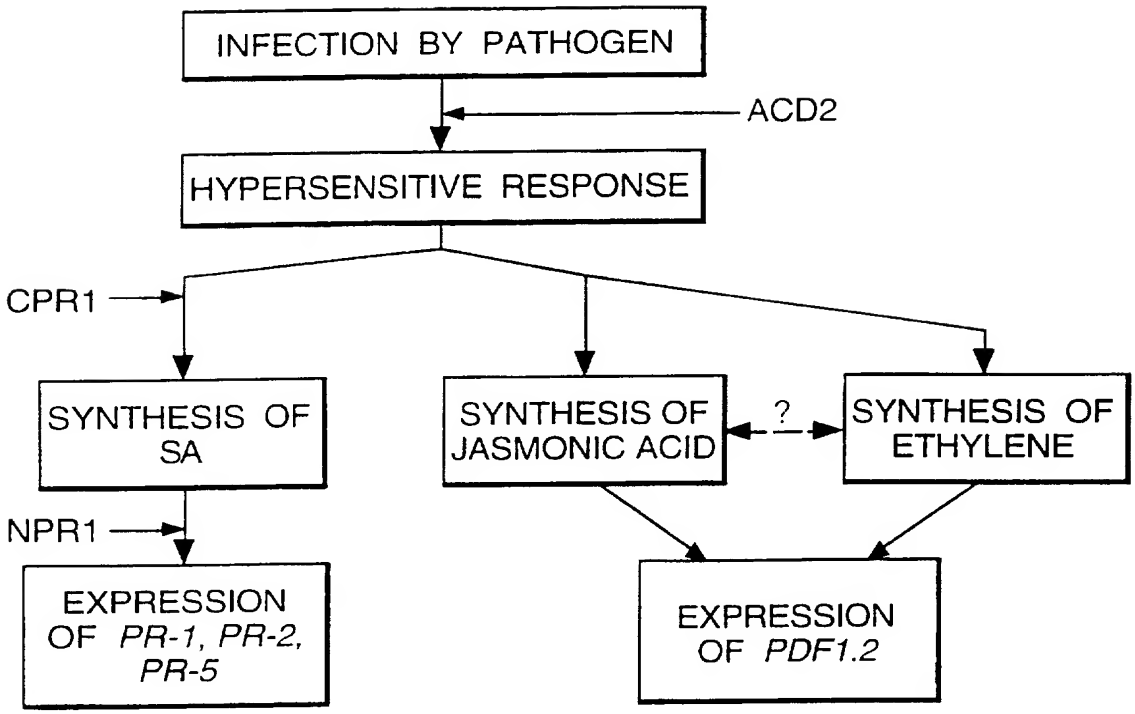

INFECTION BY PATHOGEN
ACD2
HYPERSENSITIVE RESPONSE
CPR1
SYNTHESIS OF SA
NPR1
EXPRESSION OF PR-1, PR-2, PR-5
SYNTHESIS OF JASMONIC ACID
?
SYNTHESIS OF ETHYLENE
EXPRESSION OF PDF1.2

Fig.10.

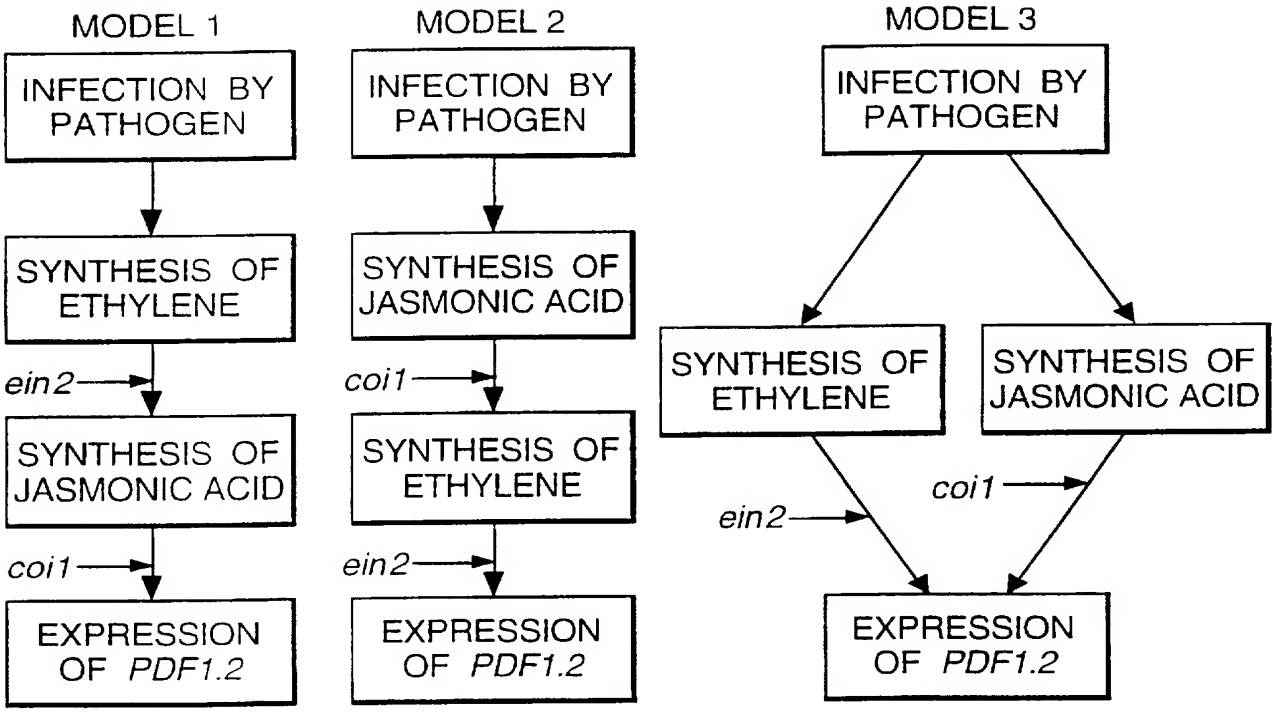

MODEL 1
INFECTION BY PATHOGEN
SYNTHESIS OF ETHYLENE
ein2
SYNTHESIS OF JASMONIC ACID
coi1
EXPRESSION OF PDF1.2
MODEL 2
INFECTION BY PATHOGEN
SYNTHESIS OF JASMONIC ACID
coi1
SYNTHESIS OF ETHYLENE
ein2
EXPRESSION OF PDF1.2
MODEL 3
INFECTION BY PATHOGEN
SYNTHESIS OF ETHYLENE
SYNTHESIS OF JASMONIC ACID
ein2
coi1
EXPRESSION OF PDF1.2

Fig.11.

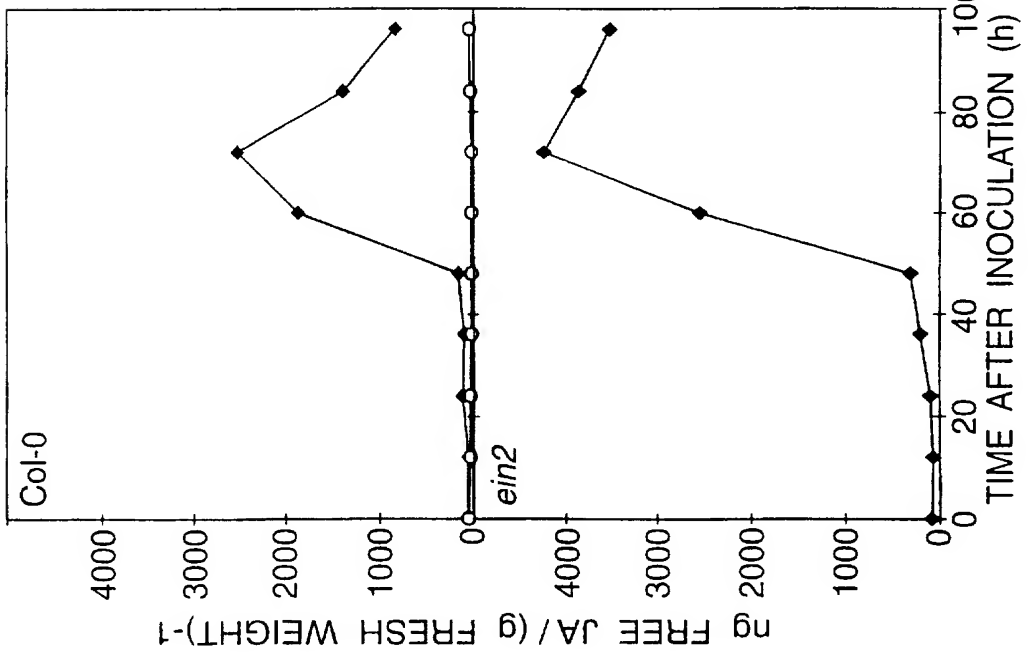

Col-0
ein2
ng FREE JA / (g FRESH WEIGHT)-1
4000
3000
2000
1000
0
0
20
40
60
80
100
TIME AFTER INOCULATION (h)

Fig.12.

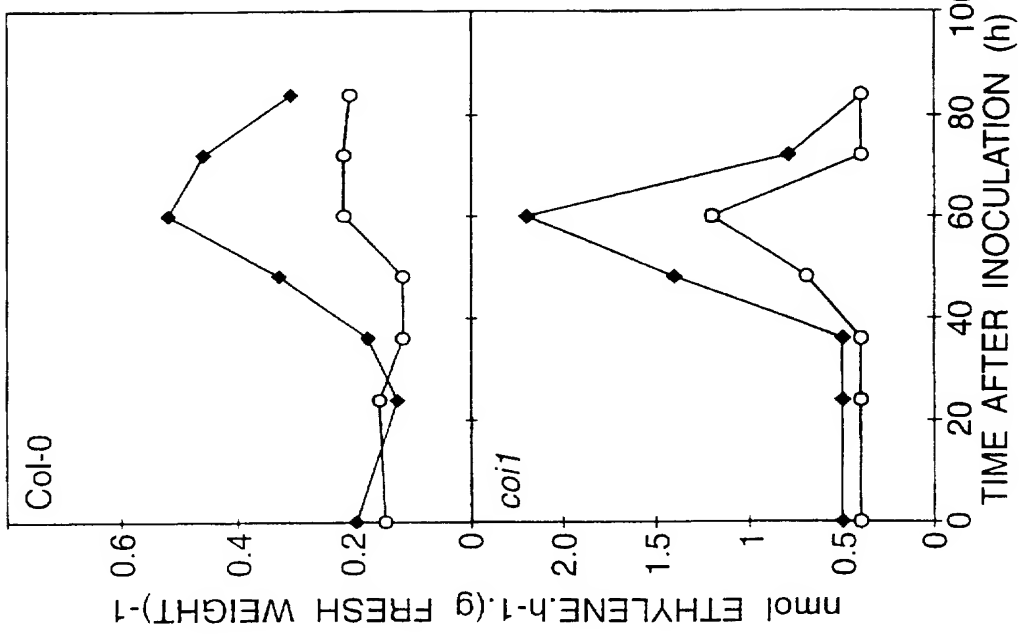

Col-0
coi1
nmol ETHYLENE.h-1.(g FRESH WEIGHT)-1
0.6
0.4
0.2
0
2.0
1.5
1
0.5
0
0
20
40
60
80
100
TIME AFTER INOCULATION (h)

Fig.13.

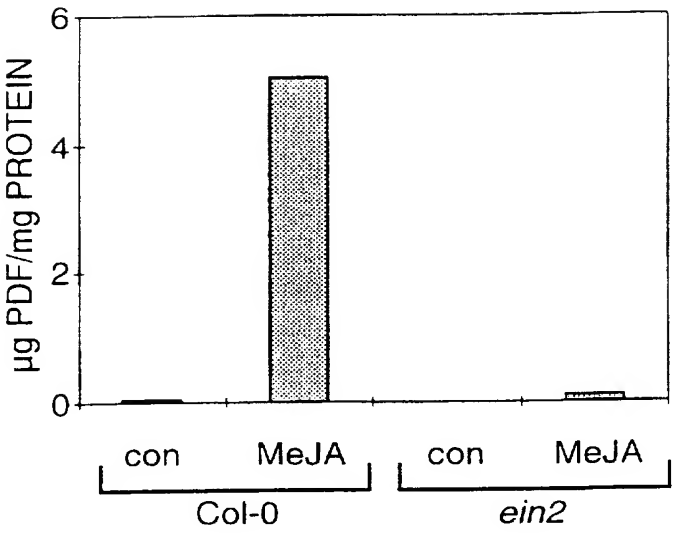


Fig.14.

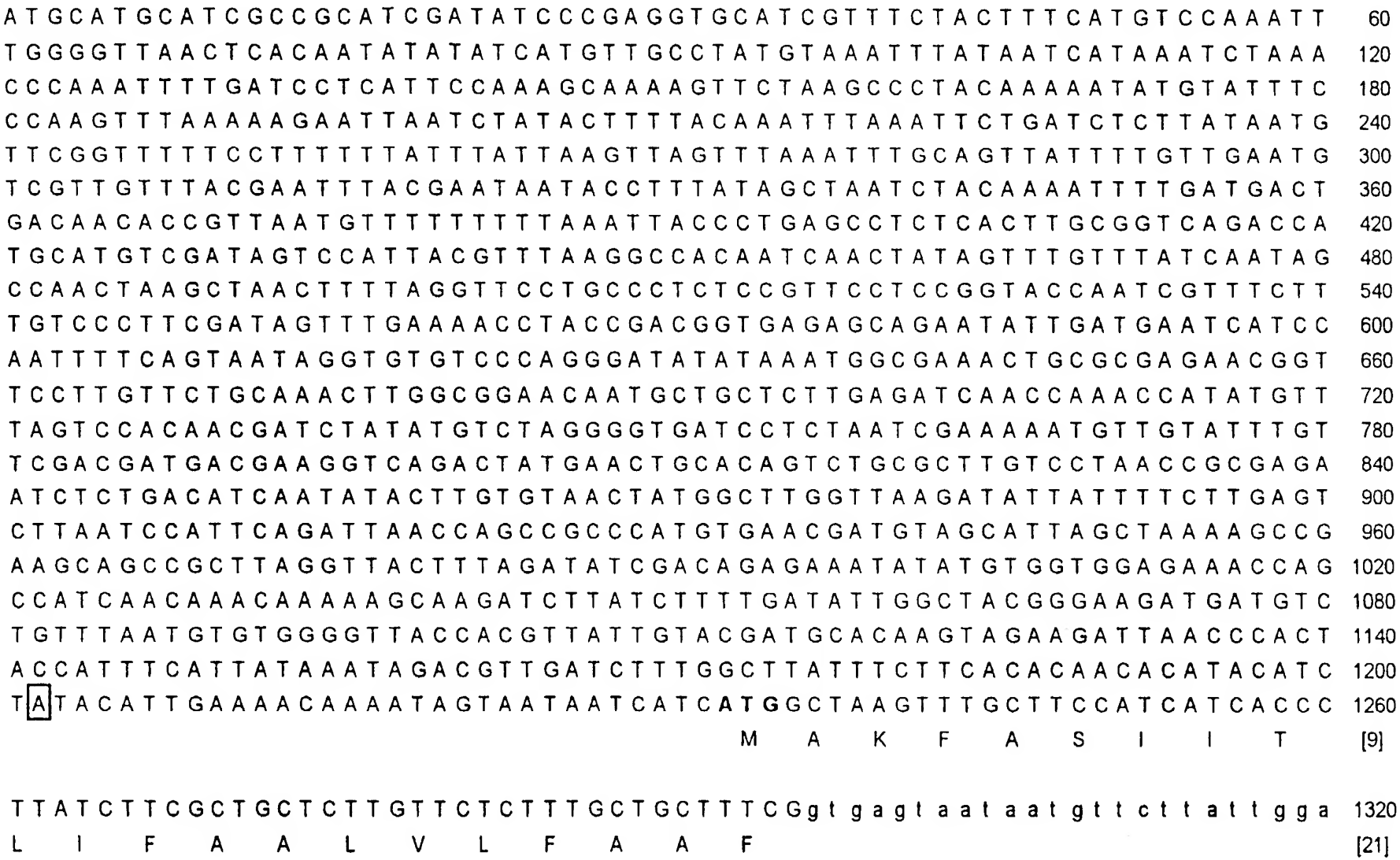


Fig.14 (Cont).

```
aatgtgttttaaatgtaatttttagttttatttgataatttattcttgactgatgtatgcatat 1380

atatgcagACGCACCCGGCAATGGTGGAAGCACAGAAGTTGTGCGAGAAGCCAAGTGGGAC 1440
         D   A   P   A   M   V   E   A   Q   K   L   C   E   K   P   S   G   G   T  [39]

ATGGTCAGGGGGTTTGCGGAAACAGTAATGCATGCAAGAATCAGTGCATTTAACCTCGAAGG 1500
   W   S   G   G   V   C   G   N   S   N   A   C   K   N   Q   C   I   N   L   E   G  [59]

AGCCAAACATGGATCATGCAACTATGTCTTCCCAGCACACAAGTGTATCTGTTACGTCCC 1560
   A   K   H   G   S   C   N   Y   V   F   P   A   H   K   C   I   C   Y   V   P  [79]

ATGTTAAATCTACCAATAATCTTTTGGTGCTAAATCGTGTGTATTTTTACATAAAAAT      1616
   C   *                                                          [80]
```

Fig.15.

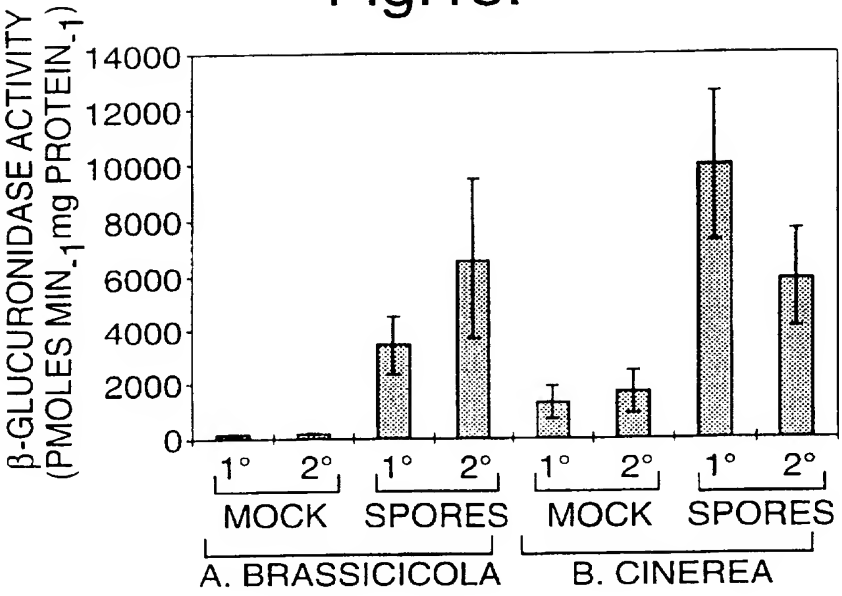

β-GLUCURONIDASE ACTIVITY
(PMOLES MIN.-1 mg PROTEIN.-1)
14000
12000
10000
8000
6000
4000
2000
0
1° 2° 1° 2° 1° 2° 1° 2°
MOCK SPORES MOCK SPORES
A. BRASSICICOLA
B. CINEREA

Fig.16.

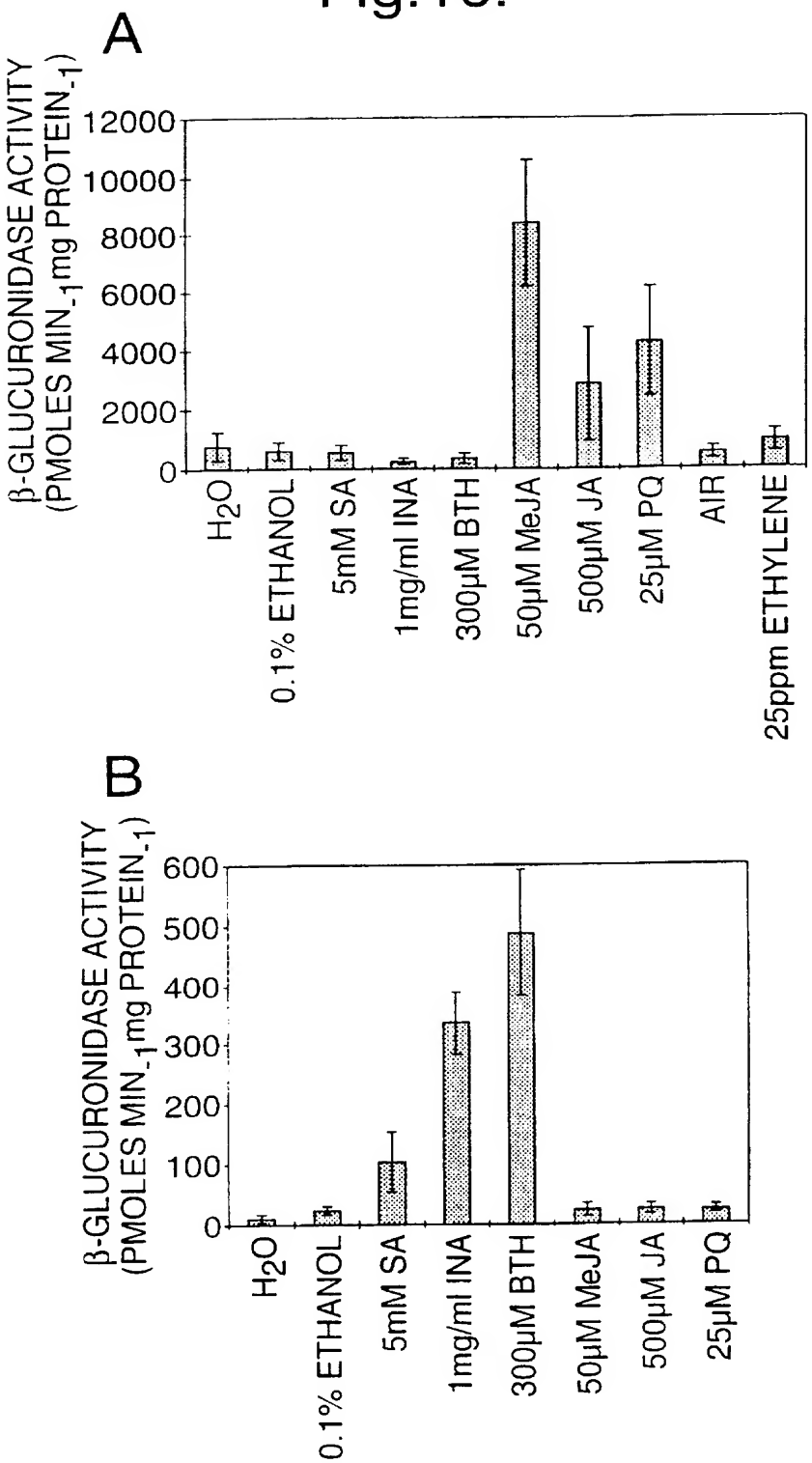

A
β-GLUCURONIDASE ACTIVITY (PMOLES MIN-1mg PROTEIN-1)
12000
10000
8000
6000
4000
2000
0
H2O
0.1% ETHANOL
5mM SA
1mg/ml INA
300µM BTH
50µM MeJA
500µM JA
25µM PQ
AIR
25ppm ETHYLENE
B
β-GLUCURONIDASE ACTIVITY (PMOLES MIN-1mg PROTEIN-1)
600
500
400
300
200
100
0
H2O
0.1% ETHANOL
5mM SA
1mg/ml INA
300µM BTH
50µM MeJA
500µM JA
25µM PQ

Fig.17.

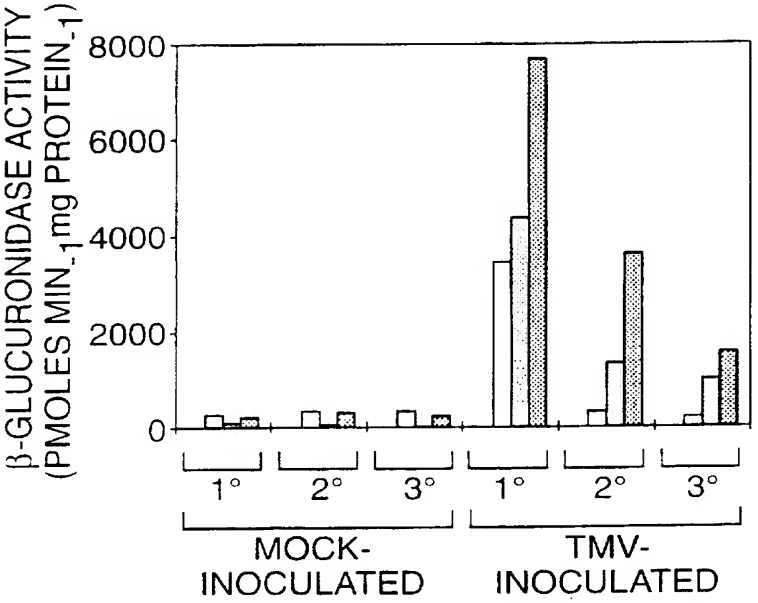

β-GLUCURONIDASE ACTIVITY
(PMOLES MIN-1mg PROTEIN-1)
8000
6000
4000
2000
0
1°
2°
3°
1°
2°
3°
MOCK-
INOCULATED
TMV-
INOCULATED

Fig.18.

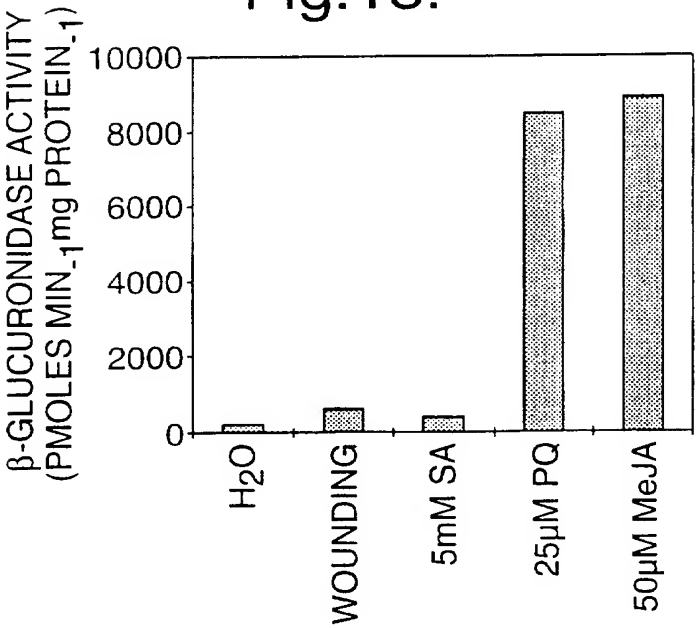

β-GLUCURONIDASE ACTIVITY
(PMOLES MIN-1mg PROTEIN-1)
10000
8000
6000
4000
2000
0
H2O
WOUNDING
5mM SA
25μM PQ
50μM MeJA

Fig.19.

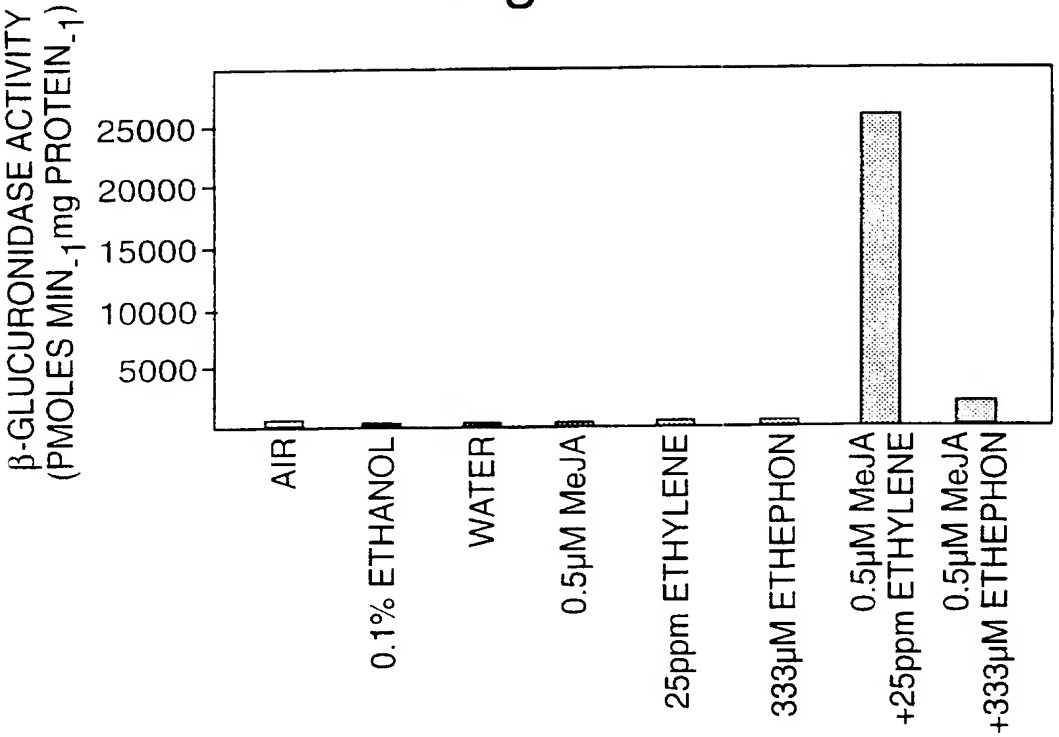

β-GLUCURONIDASE ACTIVITY
(PMOLES MIN-1 mg PROTEIN-1)
25000
20000
15000
10000
5000
AIR
0.1% ETHANOL
WATER
0.5μM MeJA
25ppm ETHYLENE
333μM ETHEPHON
0.5μM MeJA +25ppm ETHYLENE
0.5μM MeJA +333μM ETHEPHON

Fig.20.

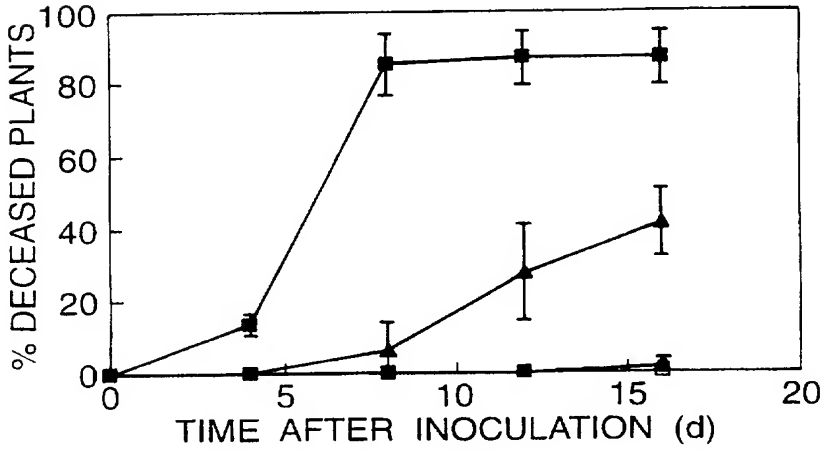

% DECEASED PLANTS
100
80
60
40
20
0
0
5
10
15
20
TIME AFTER INOCULATION (d)

Fig.21.